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INTERACTION WITH CD9 REGULATES CELLULAR ADHESION WITHIN THE B
CELL LINEAGE

BY



ANNA MASELLIS-SMITH

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of
the requirements for the degree of Doctor of Philosophy

in

MEDICAL SCIENCES (MEDICINE)

EDMONTON, ALBERTA

SPRING, 1993



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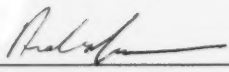
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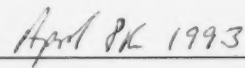
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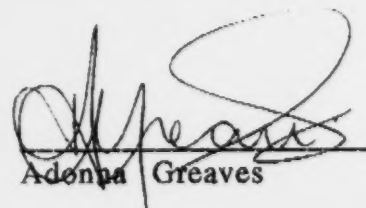
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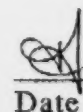
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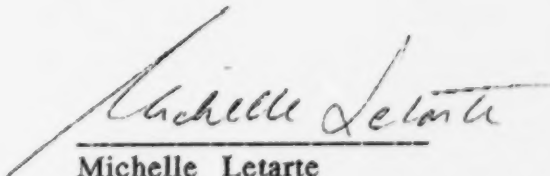
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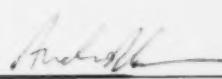
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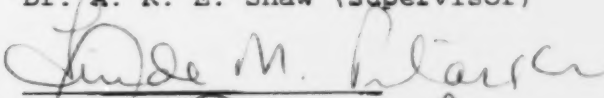
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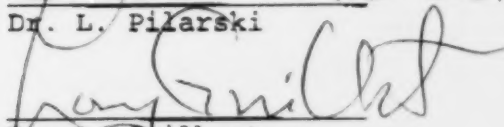
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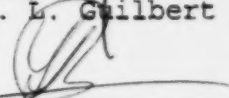
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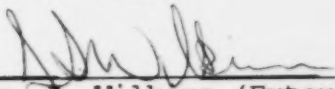
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To my parents, my loving husband and children.

ABSTRACT

CD9 is a member of the four-pass transmembrane supergene family and a potent agonist of platelet aggregation, suggesting a role in the modulation of adhesion. The goal of this study was to determine if modulation of CD9 with anti-CD9 mAbs could also induce adhesive events in B lymphoid cells.

Three CD9 mAbs, 50H.19, BA-2 and ALB₆ were found to promote rapid and irreversible homotypic aggregation of CD9-positive pre-B lymphoblastoid cell lines NALM-6 and HOON. The anti-CD9 mAb induced adhesion requires the expenditure of metabolic energy and a functioning cytoskeleton, and is not inhibited by Arg-Gly-Asp-Ser peptide. As HOON is negative for β_2 integrins, and NALM-6 is β_2^{lo} , β_2 integrins may not be the mediators of CD9-induced aggregation. Investigation of the expression of very late activation (VLA) integrins in five human leukemic cell lines of pre-B cell phenotype indicates that $\alpha_4\beta_1$ is the dominant integrin in all five, three possessed $\alpha_5\beta_1$ and one $\alpha_6\beta_1$. Anti-VLA- α_4 , and anti-VLA- β_1 mAbs induced strong aggregation which was not blocked by the anti-Fc γ IIIR mAb IV.3. However, aggregation was effected in only three of the five lines suggesting the involvement of molecules other than VLA-4. The level of expression of CD9, but not that of CD11a, CD18, CD19, CD44, or CD54 was found to correlate with the level of aggregation. Investigation into the role of CD9 in interactions between pre-B cell lines and bone marrow cultures indicates that CD9 is involved in promoting heterotypic cell-cell interactions. Anti-CD9 mAbs ALB₆ and 50H.19 and 50H.19 F(ab')₂ enhanced the adherence of the pre-B ALL cell line NALM-6 and HOON to bone marrow-derived fibroblasts. Anti-CD9 enhanced adherence was not blocked by the anti-Fc γ IIIR mAb IV.3. Utilizing blocking mAbs recognizing β_1 integrins, anti-CD9 mAb enhanced adhesion was found to be dependent upon the upregulation of $\alpha_4\beta_1$ and $\alpha_5\beta_1$. Synthetic peptides homologous to the CCB_D and CS-1 region of fibronectin, and anti-fibronectin mAbs reduced anti-CD9 mAb enhanced adhesion, indicating the selective enhancement of avidity for fibronectin. Evidence is presented that anti-CD9 mAbs can also induce the development of a dendritic-like cell from a subpopulation of transiently adherent mononuclear cells of human peripheral blood. The transiently adherent cells include a major CD9 positive subpopulation bearing the B lineage markers CD19 and CD20, but lacking markers of the monocyte and T lineages. Two anti-CD9 mAbs, 50H.19 and ALB₆, as well as the F(ab')₂ of 50H.19, promoted development of cells with dendritic morphology by 72 hours of culture. Dendritic cell differentiation was blocked by soluble RGD peptide, and by mAbs against VLA-5 (α_5), β_1 and CD44, again suggesting that CD9 may regulate the interaction of VLA-5 with its substrate.

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LIST OF SYMBOLS AND ABBREVIATIONS

DC:	DENDRITIC CELL
ECM:	EXTRACELLULAR MATRIX
FN:	FIBRONECTIN
IIICS:	TYPE III CONNECTING SEGMENT OF FIBRONECTIN
HA:	HYALURONIC ACID
HRP:	HORSERADISH PEROXIDASE
S.I.:	STIMULATION INDEX
BM:	BONE MARROW
BM-AD:	BONE MARROW ADHERENT CELLS
BM-FB:	BONE MARROW FIBROBLASTS
VCAM:	VASCULAR CELL ADHESION MOLECULE
CFU-S:	COLONY FORMING UNIT-SPLEEN
FCS:	FETAL CALF SERUM
PBS:	PHOSPHATE BUFFERED SALINE
MAB:	MONOCLONAL ANTIBODY
ICAM:	INTERCELLULAR ADHESION MOLECULE
HBSS:	HANKS BALANCED SALT SOLUTION
LFA:	LYMPHOCYTE FUNCTION-ASSOCIATED ANTIGEN
VLA:	VERY LATE ANTIGEN
RGDS:	ARG-GLY-ASP-SER
RGES:	ARG-GLY-GLUTAMIC ACID-SERINE
ALL:	ACUTE LYMPHOBLASTIC LEUKEMIA
CALLA:	COMMON ACUTE LYMPHOBLASTIC LEUKEMIA ANTIGEN
CD:	CLUSTER DESIGNATION

I: INTRODUCTION

I-I. Introduction to CD9 and the four-pass transmembrane family:

A. *DISCOVERY OF CD9*

The development of the immune system (hematopoiesis) arises from a common multipotential stem cell which has the capacity to give rise to multiple lineages of cells with diverse interrelated functions. Although other organs can support hematopoiesis including the spleen (1) and liver (2), the bone marrow appears to provide the optimal microenvironment for the regulation, differentiation and expansion of the various hematopoietic lineages. The bone marrow microenvironment is capable of supporting such development primarily due to the presence of bone marrow stromal elements and soluble mediators, produced by both the stromal elements and progenitor cells. These factors act in combination to regulate the differentiation and proliferation of hematopoietic precursors. The identification of components on the cell surface has allowed for the classification of hematopoietic cell lineages, as well as allowing the assignment of differentiation stages within lineages. The function of cells within the various lineages is also, in part, dictated by the composition of receptors on the cell surface. Expression of certain receptors at various stages of cellular differentiation endow the cell with the capacity to interact with and respond to a challenging microenvironment.

Cell surface markers were initially identified by the ability of certain cells to perform certain functions; for example, T cells were initially identified by their ability to form rosettes with erythrocytes (3). Monoclonal antibodies are now used as a research tool to characterize a variety of biological functions. The use of mAbs has simplified the purification of surface receptors, which has greatly enhanced their characterization both biochemically and functionally. Furthermore, mAbs directed against cell surface receptors has allowed for a

more detailed analysis of cell types within the hematopoietic system. Indeed, the classification of human leucocyte antigens within the CD (cluster of differentiation) system was initially based on the classification of monoclonal antibodies and the antigens they recognize (4). In general, antigens are assigned to a particular cluster after it has been recognized by greater than one antibody, and a molecular weight is determined. Techniques which employ the use of mAbs in antigen recognition include immunofluorescent cell surface labelling, immunoprecipitation, and immunohistology on tissue sections.

Using conventional hybridoma technique, Kersey et al (5) produced mAb BA-2 in response to a non-T, non-B common acute lymphoblastic leukemia (cALL) cell line. BA-2 recognized a polypeptide of 24,000 Mr by SDS-PAGE analysis, termed p24. Indirect immunofluorescence analysis using mAb BA-2 suggested that the distribution of p24 included <1% peripheral blood cells, 3% bone marrow mononuclear cells (40-60% TdT+), > 50% non-T, non-B cALL with high reactivity, and 18% CLL with low reactivity. Other laboratories have since produced a variety of mAbs recognizing a similar polypeptide. These mAbs have been grouped together, based on criteria established at the Fourth (4th) International workshop and conference on human leucocyte differentiation antigens (6), into cluster designation 9, or CD9. The characteristics of anti-CD9 mAbs are: a) recognition of various cell types within and without the hematopoietic system b) the ability to promote platelet aggregation c) immunoprecipitation of a 24,000 Mr polypeptide and immunoblotting of the same size antigen.

B) CD9 TISSUE DISTRIBUTION

BI. CD9 expression on hematopoietic cells

The use of anti-CD9 mAbs to determine CD9 tissue distribution has indicated a broad distribution which includes various hematopoietic as well as nonhematopoietic cells. Within hematopoietic lineages, CD9 is strongly expressed on platelets (7), and is also expressed on basophils (8), eosinophils (9), and mast cells (10). The expression of CD9 on blood monocytes is controversial. Indirect immunofluorescence with anti-CD9 mAbs indicates an extracellular staining pattern which suggests that the antigen may be acquired from surface-bound platelets (11). Cells of lymphoid origin, both T and B, express CD9. Although CD9 is not found on pluripotent progenitors (CFU-GEMM) or committed progenitors (CFU-E, CFU-GM, BFU-E) (12), pre-B cells ($TdT^+CD10^+CD20^-$) within bone marrow are CD9 positive (12), and 42% of circulating B cells weakly express CD9 (13). Similarly, a subpopulation of activated B cells strongly express CD9 (14). Within the T cell lineage multi-negative thymocytes and thymocytes located in the medulla express CD9 (15), while the majority (greater than 90%) of circulating T cells are negative (16 and S. Shav, personal communication). Upon activation, both CD4+ and CD8+ T cells express CD9 (17).

BII. CD9 expression on nonhematopoietic tissue

Based on immunohistochemical analysis of tissue, CD9 expression is widely diversified. It has been found on osteoclasts and foreign body giant cells (18), both which are multinucleated cells formed by a process involving cell fusion. Most epithelial cells from a variety of tissues stain positive, which includes expression on mucosal epithelium of the intestine, ductal epithelium and myoepithelium of breast and salivary glands, red pulp epithelium of spleen and thymus, squamous epithelium of tonsil epithelium, as well as distal tubules and Bowmans capsule within the kidney (19). CD9 has been described on smooth muscle, but is absent from skeletal muscle. Anti-CD9 mAbs have also been shown to stain the interstitial spaces of various tissue sections. It was suggested that these

mAbs may stain an extracellular domain on keratin since anti-keratin mAb immunoprecipitates were reactive with anti-CD9 mAbs on western blotting (20). CD9 expression on the various tissues may reflect different states of cell differentiation and activation. Furthermore, evidence from gene cloning and functional studies in platelets indicate that CD9 is capable of signal transduction, and may possibly regulate ion channels directly or indirectly (see further discussions). This may be relevant to many of the CD9⁺ cells (ie: osteoclasts, and peripheral nerve cells) whose functions are particularly sensitive to the ion levels.

C. CD9 expression on malignant cells.

CI. Hematopoietic malignancies.

Hematopoietic malignancies are often considered malignant counterparts of normal cells. Various techniques, such as expression of cell surface markers, morphology, and molecular biology, have been used to assign hematopoietic malignancies within a given lineage and stage of differentiation. The study of malignant cells in comparison to their normal counterparts have increased understanding of the malignant process. Such investigations has led to the discovery of certain elements which have been introduced or altered, within the malignant cell (21). These would include activation of cellular oncogenes, chromosomal expansion, chromosomal deletion, and altered cell surface components. The study of cell surface proteins upon the malignant cell not only serves to establish the lineage of a particular malignancy but also, if the function of the surface protein is characterized, to augment understanding of the course of the malignancy.

CII. Clinical use of anti-CD9 mAbs

The absence of CD9 on the multipotential and committed progenitors of hematopoietic cells makes this antigen a good candidate for use in mAb-mediated bone marrow purging of malignant cells during autologous bone marrow transplantation (BMT). Autologous BMT is a widely accepted procedure, particularly in the absence of HLA-matched donors for allogeneic BMT. During autologous BMT, bone marrow cells harvested from patients in remission are purged of leukemic cells by the use of monoclonal antibodies which recognize tumor associated antigens, in combination with toxic pharmacological agents (22). The observation of Stepan et al. showed that anti-CD9 mAb BA-2 effectively lysed up to 70% fresh leukemic cells in the presence of human or rabbit complement (23), indicating that anti-CD9 mAbs were potentially effective immunotherapeutic agents. The efficacy of antibody induced leukemic cell lysis was then increased by using a combination of antibodies directed against CD24, CD9 and CD10 (24). Although the combination of these antibodies increased killing of leukemic cells, normal bone marrow pluripotent and monopotent progenitor cell were not harmed. These results indicated that CD9, and other antibodies could be used as immunotherapeutic agents without compromising the reconstitution of normal bone marrow cells.

The major obstacle in autologous BMT is recurrence of leukemia. Despite pretransplant therapy, and techniques to purge autografts of residual leukemic cells, some patients in complete remission do relapse. As CD9 is strongly expressed on hematopoietic malignancies, particularly nonT, nonB ALL, it has been implicated as a useful clinical tool to chart the progression of the disease. Indeed, Miguel et al indicated that the presence of CD9 on acute myeloblastic leukemia (AML) cells had an adverse effect on complete remission and survival of patients (25). Two studies by Komada et al. indicate that the shedding of CD9 by leukemic cells can be used to detect residual leukemic cells post-treatment, and to predict patient relapse. These investigations utilized an indirect ELISA technique in which CD9 bound to the lectin *Ricinus communis* agglutinin is quantitated by

anti-CD9 mAb. The first study showed that the CD9 antigen was isolated from the cerebral spinal fluid (CSF) of CD9⁺ ALL patients with central nervous system (CNS) relapse, but not from patients without CNS relapse, or from patients in complete remission (26). The levels of CD9 in CSF were high in these patients prior to cytologically proven CNS involvement, but returned to undetectable levels after initiation of chemotherapy. These findings suggested that the identification of CD9 antigen in CSF could be used as a primary indicator of CNS relapse. A second study following the shedding of CD9 from bone marrow cells of ALL patients in morphological remission indicated that the shedding of CD9 during relapse correlated with poorer prognosis, and early relapse (27). These findings suggest that detection of CD9 could be used as a clinical tool in patient monitoring, and establishing treatment protocols.

D. Biochemical characterization

DL: Three different molecular weight forms

CD9 was first described as a protein of 24,000 dalton protein on pre-B cells. However, in most cell lines in which immunoprecipitation has been carried out, anti-CD9 mAbs immunoprecipitates a major 22,000 dalton, as well as a minor 24,000 dalton form (28). Pulse-chase analysis indicates that all the various forms of CD9 arise from a nonglycosylated polypeptide precursor of 20,500 daltons (29). The differences in molecular weight observed for CD9 are due to differences in glycosylation, and from several lines of evidence, expression of these various molecular weight forms is cell-type dependent and may reflect expression of CD9 with specific functions. On platelets, CD9 is 22,000, 24,000 and 27,000 dalton. However, on carcinoma cell lines, there is only a major 22,000 and a minor 24,000 form (30). The 22,000 form of CD9 (gp22) may contain O-linked glycosylation, as it is sensitive to treatment with alkaline agents, is

resistant to tunicamycin treatment (a potent inhibitor of N-linked glycosylation) and furthermore incorporates ^3H -galactose, a sugar most commonly added during the step-wise addition of sugar moieties which occurs in O-linked glycosylation. The 24,000 dalton form of CD9 contains both O-linked and N-linked glycosylation, as it is sensitive to tunicamycin, and endoglycosidase-F (specific for N-linked carbohydrates), and incorporates ^3H -mannose (not found in O-linked carbohydrate chains), as well as ^3H -galactose. The expression of more than one molecular weight form of CD9 on a given cell may reflect a multifunctional role for CD9.

DII. CD9 is variably acylated

Long chain fatty acid modification has been reported on a number of proteins.(31-33), The majority of transmembrane proteins contain the 16-carbon fatty acid, palmitic acid, while other proteins have been shown to contain the 14-carbon fatty acid myristic acid. These fatty acids reportedly interact with the portion of the protein which interacts with the lipid bilayer, and are attached by either ester (palmitic acid), or amid (myristic acid) linkages. Fatty acid ligation may serve in transport of glycoprotein from the Golgi complex, in anchoring and stabilizing proteins within the plasma membrane, and facilitating interactions with other plasma membrane proteins. Acylation of proteins by transacylases predominantly occurs shortly after protein synthesis, although protein acylation can occur in the absence of known protein synthesis. CD9 has been shown to contain long chain fatty acids. Seehafer et al. (34) reported all three forms of CD9 were acylated, and further implicated CD9 as the major fatty acid ligated platelet protein. This finding suggests that CD9 acylation may occur in the absence of protein synthesis possibly by transacylases compartmentalized elsewhere than within the Golgi. LeBien et. al. (35) suggested that fatty acids may be linked to CD9 by an ester linkage, as treatment of SDS gels containing ^3H -palmitic acid-CD9 resulted in loss of label. Seehafer et. al. reported the enzymatic

incorporation of palmitic acid into gp22 and gp24 proteins on intact cells. Seehafer et. al. (36) further reported the incorporation of ^3H -myristate into CD9. The inability of hydroxylamine to remove ^3H -palmitic acid label from platelets confirmed the ester-linkage of fatty acids on CD9 reported earlier by LeBien. Moreover, CD9 has been shown to contain multiple acylation sites by the incorporation of palmitic acid into two peptides localized to the membrane proximal region of the antigen. The localization of ester-linked fatty acids to this site suggests that fatty acids play a role in the anchorage and perhaps antigen conformation within the plasma membrane.

E. Cloning of CD9

EI. Structure and topology of CD9

EI. A. Methods used to clone CD9

To understand the biochemical and molecular properties of a given protein would enhance the understanding of its biological function. Gene cloning allows for the prediction of amino acid sequences, as determined from DNA sequences. The amino acid sequence of a given protein potentially provides information on tertiary structure of proteins within membranes, and provide information on protein function by comparing sequences with other proteins with known functions. To this end, the gene encoding CD9 was cloned independently by two groups, and the predicted amino acid sequence has given insight into the structure and function of this protein.

In the two studies described, complementary DNA sequences (cDNA) were obtained from cDNA libraries compiled from cells known to express CD9. In the first report, a megakaryocyte library was screened with an assortment of CD9 mAbs to obtain clones

which positively expressed CD9 (37). In the second report, a polymerase chain reaction (PCR) product obtained from purified CD9 peptide fragments was used to obtain CD9 positive clones from both a fibroblast and an endothelial cell library (38). That each group positively isolated cDNA which encoded for CD9 was determined by the following criteria: 1) identification of cDNA clones with anti-CD9 mAbs 2) size of the protein 3) comparison of sequences with that obtained from the purified protein and 4) the cDNA clone expressed a protein in oocytes and CHO cells reactive with a known anti-CD9 mAb.

EI. B. CD9 contains four membrane spanning sequences

The predicted amino acid sequence obtained by both groups indicates a protein with four hydrophobic sequences, which is in contrast to the earlier findings of Newman et al.(39) who reported CD9 as a nonintegral membrane protein inaccessible to label with a lipophilic nitrene reagent. That the four hydrophobic sequences represent hydrophobic domains is suggested by the following: 1) each hydrophobic domain is 24-28 amino acids in length, which is sufficient to span the membrane and 2) each sequence is bound by a charged amino acid characteristic of hydrophobic membrane spanning domains.

EI. C. CD9 contains both amino-terminal and carboxy-terminal ends oriented towards the cytoplasm

The orientation of CD9 within the plasma membrane, as suggested by the amino acid sequence, is represented in Figure 1 (40). The hydrophilic domains of CD9 were determined by hydropathicity plot of the amino acid sequence of CD9. Furthermore, from the predicted amino acid sequence, CD9 contains an N-glycosylation site at the NH₂-terminus at position 52, suggesting that this site represents an extracellular domain. The amino and carboxy termini of CD9 are both oriented on the inner side of the plasma

membrane. That the NH₂-terminus is intracellular is suggested by the lack of leader sequence, and the presence of a hydrophobic sequence which begins only 12 amino acids upstream from this end. It is suggested that the first hydrophobic sequence acts as the leader sequence allowing for the integration of the protein into the plasma membrane.

Figure EI. C1

EI. D. CD9 has structural homology to ion channels

Interestingly, the structure of CD9 is reminiscent of the structure of certain ion channels. Whether CD9 may be a component of a multi-subunit ion channel is yet to be determined. However, the anti-CD9 mAb ALB6 increased the incorporation of ⁴⁵CaCl₂ into platelet membrane vesicles (41). Similarly, the addition of ALB6 to the pre-B cell line increased intracellular calcium levels, as indicated by an increase in fluorescence intensity using the calcium sensitive reagents Fura-2 and Fluo-3 (42). These results suggest that CD9 may be a component of a calcium channel, or may regulate the activity of such a channel.

FI. CD9 structural homology with a new family of proteins

On the basis of amino acid sequence, CD9 was shown to have sequence identity, as well as structural homology with several proteins (Table I) (40). The members of this family are proteins which have identical structure. Each protein possesses four putative transmembrane domains and two hydrophilic domains, with amino and carboxy termini oriented on the cytoplasmic face of the plasma membrane. This family of proteins has been termed four-pass transmembrane protein family due to their multiple membrane spanning domains. The highest degree of homology between members of this family exists within the first three hydrophobic domains, which may reflect a functional conservation. The greatest degree of variability exists among the two hydrophilic domains, suggesting that

these domains may be involved in differences such as ligand binding. Other highly conserved regions include six cysteine residues and their flanking sequences. These cysteine residues may form disulfide bonds across a short distance (as the molecular weight does not alter upon reduction), and could play a role in the stabilization of the molecule. Further variation exists within the number of potential N-glycosylation sites within a given member of this family. While most members of this family are heavily glycosylated, TAPA-1, SM-23, CO-029 and CD9 appear to not be glycosylated or contain only one putative N-glycosylation site located within either in the first or second extracellular domain. This differences in glycosylation patterns may reflect a cell specific function such as ligand binding, while the hydrophobic domains of these proteins may be functionally conserved for the proteins structural integrity and for possible interactions with other plasma membrane proteins.

TALBE FI

FII. Expression and function of the tetra-spans transmembrane protein family:

Members of the tetra-spans transmembrane supergene family are expressed on various cell types, and at various stages of differentiation. The biological function of CD9 family members are unknown, however, studies with mAbs have given some clues as to the function of these molecules. MAb interaction with receptors may mimic the interaction with a natural ligand, may block interaction with a natural ligand, or may induce conformational changes in receptor structure to render it active. The results from studies utilizing mAbs against the various tetra-spans family members suggests that these antigens may play a role in differentiation and adhesion. The protein sequence of many proteins within this family is conserved among different species, suggesting that these proteins play an important biological function. The following discussion will present information on

members of the tetra-spans family with particular emphasis on tissue distribution, structure and possible functions:

A. ME491/CD63

ME491/CD63 is a protein of 30,000-60,000 dalton, first described as a melanoma-progression antigen, as it is highly expressed during the early stages of melanoma, but lost with tumor progression (43). Treatment of CD63 with endoglycosidase F reveals a protein core of 20,000 daltons, suggesting that CD63 is a highly glycosylated protein (44). Indeed, analysis of CD63 by SDS-PAGE indicates a ladder of proteins, which may reflect various glycosylated forms of the antigen. CD63 expression is largely confined to lysosomal granule membranes of nonactivated cells, while surface expression increases up to 100 fold after cell activation (45). Although CD63 does not share sequence homology with other lysosomal membrane proteins, such as LAMP-1 and LAMP-2, it does contain a consensus dipeptide sequence (Gly-Tyr) in its cytoplasmic tail which is responsible for targeting proteins to the lysosome (46). Despite its ubiquitous lysosomal expression, CD63 is weakly expressed on the surface of many nontransformed cells and tissue (Dr. W. Dixon personal communication). Although the biological role of CD63 is unknown, mAbs against CD63 inhibit phorbol ester induced monocyte binding to serum coated surfaces (47) and inhibit T and B lymphocyte aggregation (48), suggesting a role in cell adhesion. Furthermore, given the presence of extensive carbohydrate moieties, CD63 may function to protect of lysosomal, and plasma membranes from enzyme degradation. Other studies indicate that CD63 is capable of internalization and localization to the nucleus where it bound to a 55Kd chromatin protein and inhibits transcription of rRNA genes (49), suggesting a possible role for CD63 is the regulation of gene translation.

B. TAPA-1

While screening antibodies able to disrupt cell growth, Oren et al. characterized an antigen recognized by mAb 5A6. MAb 5A6 reacted with most human cell lines tested including lines of hematolymphoid, neuroectodermal and mesenchymal origin (50). Furthermore, mAb 5A6 significantly inhibited proliferation of many human cell lines, particularly lymphoid cell lines. Consequently the antigen was named TAPA-1, or target of an antiproliferative antibody. TAPA-1 is a 26,000 which is sparsely glycosylated, containing a potential glycosylation site adjacent to the carboxy terminal end. TAPA-1 does contain a potential N-myristylation site, possibly at the N-terminal glycine. The ability of mAb 5A6 to inhibit proliferation of cell lines suggests that TAPA-1 may be involved in regulation of cell proliferation. Although anti-TAPA-1 mAb 5A6 reacted similarly with most lymphoid and nonlymphoid cell lines, the ability of mAb 5A6 to inhibit proliferation was variable. This suggested that the expression of TAPA-1 was necessary but not sufficient to render the cells sensitive to inhibition by mAb 5A6, and that an associate molecule may be required for transmission of a negative signal initiated by binding of anti-TAPA-1 mAbs. Indeed, TAPA-1 was found to be physically and functionally associated with the Leu-13 antigen (51). MAbs against both TAPA-1 and Leu-13 induce homotypic aggregation of cell lines, as well as induce antiproliferative effects in lymphoid cells. The anti-proliferative effect of the two mAbs were not additive, suggesting that the two antigens may share a common effector pathway. MAb 5A6 immunoprecipitation of ¹²⁵I labelled cell lines revealed a 26,000 dalton protein (TAPA-1) as well as a 16,000 antigen reactive with mAbs against Leu-13. Furthermore, that the two antigens are functionally associated is reflected by the ability of antibodies against either TAPA-1 or Leu-13 to modulate the expression of both antigens.

C. IA4/R2

Searching for antigens involved in LAK cytotoxicity of target cells, Gil et al (52) produced a mAb, IA4, which was shown to have almost identical amino acid sequence homology with a previously described mRNA species (identified from Jurkat cells) encoding the putative inducible protein R2, a member of the tetra spans protein family. IA4 mAb reacted strongly with both pre-B cell and mature B cell lines, as well as monocytic and NK cell lines. While mAb IA4 reacted weakly with peripheral blood cells (B cells, T cells and large granular lymphocytes), the expression of the IA4/R2 antigen increased upon stimulation of cells with the mitogen PHA. Furthermore, treatment of U937 or Daudi cell lines with a combination of tumor necrosis factor and IL-4 upregulated IA4. The IA4/R2 antigen appears to be a pan-activation marker with no apparent restriction to hematopoietic lineage. The function of this antigen may be to regulate cell activation and differentiation, as mAb IA4 inhibited, in a dose dependent manner, the activation of peripheral blood by the mitogen SAC. Treatment of the U937 with mAb IA4 resulted in a rapid increase of intracellular calcium, suggesting that the IA4/R2 antigen is capable of initiating the transduction of a signal across the plasma membrane which, at least in B cells, influences cell activation.

D. CD37

CD37 is a B cell associated antigen, found on normal, and malignant IgG⁺ cells, which exhibits a high degree of homology with the NH₂ terminal of the rat OX-44 antigen (53). Expression of CD37 on B cells is stage specific, as it is expressed by IgG bearing cells, but expression is lost upon terminal maturation. CD37 is a 40,000-52,000 dalton protein which exhibits extensive microheterogeneity in molecular weight, possibly due to incomplete glycosylation or digestion of carbohydrate chains by exoglycosidases (54). Indeed, CD37 is derived from a core protein of 25,000 dalton, to which two or three complex N-linked carbohydrate chains (endoglycosidase F sensitive) of various lengths are

attached. This suggests that up to 50% of the molecular weight of this antigen is comprised of carbohydrate. Analysis of expression using electron microscopy indicates that CD37 is expressed at the cell surface as well as within intracellular vesicles. In comparison with other proteins, CD37 most resembles the mannose-6-phosphate receptor in its biochemical characteristics. The mannose-6-phosphate receptor is involved in intracellular transport and surface receptor-mediated endocytosis of lysosomal enzymes. As CD37 resembles the mannose-6-phosphate receptor in its subcellular distribution, it may also function in a similar manner. Although the function is unknown, an anti-CD37 mAb has been shown to modulate the activation of B cells, suggesting that this antigen may be involved in the regulation of cell activation and differentiation.

G. Biological effects of CD9

GI. MAbs as agonists of cell activation

Many mAbs exist which are capable of cell activation through induction of intracellular signal pathways, leading to cell proliferation and/or differentiation. In these instances, the mAb is thought to mimic the natural ligand for the receptor. Ligand (mAb):receptor complexes may transmit a message across the plasma membrane triggering a conformational change of the receptor. The conformational change which occurs at the cell exterior is transformed into second messengers (effectors) on the cell interior by actions of membrane bound enzymes, such as G-proteins. Each receptor may be linked to a variety of membrane bound enzymes, which in turn are linked to multiple effector pathways which ultimately amplify the first messenger signal. The use of purified mAbs is of assistance particularly when the natural ligand is unknown, and furthers the biochemical characterization of the antigen and its signalling pathway.

Mabs against CD9 are agonistic mAb, that is, they are capable of cell activation upon binding the receptor. The binding of mAb to CD9 may itself be responsible for induction of intracellular signals, or alternatively, CD9 may be laterally associated with proteins on the cell surface which are responsible for transduction of information to the cell interior. That anti-CD9 mAb cell activation may occur through both mechanisms above is discussed further in this section.

GII. Role of CD9 in lymphocyte proliferation and activation

Cell activation is a critical step in the response of lymphocytes to antigenic or mitogenic stimuli. Activation results in changes in the cells ability to respond to another cell, as well as the surrounding microenvironment.

Studies designed to investigate the role of CD9 in cell activation and proliferation were centered around T cells. Early reports suggested that T cell proliferation induced by mitogen, alloantigens, or soluble antigens was not blocked by anti-CD9 mAb J2 (17). Peripheral blood mononuclear cells, stimulated with an anti-CD3 mAb OKT3, were also not inhibited by anti-CD9 mAbs FMC56 or FMC8 (55). Furthermore, anti-CD9 mAb DU-ALL-1 was unable inhibit, or enhance, the generation of cytotoxic lymphocytes in a mixed lymphocyte reaction (MLR), or the induction of immunoglobulin secretion after B cell stimulation with pokeweed mitogen, or a combination of IL-4, low molecular weight BCGF and anti-IgM (55, 15)..

GIII) Role of CD9 in cell adhesion

Cell adhesion plays an important role in the immune system. Adhesion is involved in events ranging from differentiation within the bone marrow (B cells) and thymus (T cells),

extravasation through high endothelial venules from peripheral blood, interaction with antigen bearing cells and subsequent activation. The ability of a cell to respond to its microenvironment is dictated by the expression of surface antigens at a given stage of differentiation.

A. Anti-CD9 mAbs enhance fibrin clot retraction

The first evidence of CD9 involvement in cell adhesion was observed in a fibrin clot retraction (FCR) assay system. Fibrin clots are formed during the early phases of wound healing, in which the migration of fibroblasts and epithelial cells within the fibrin clot contributes to the contraction and eventual healing of the wound (56). Normal fibroblasts and epithelial cells migrate into and spread within the fibrin clot. The addition of anti-CD9 mAb ALB6 greatly increased fibroblast FCR activity (57). This suggests that CD9 may be directly involved in binding to fibrin, or is involved in regulation of processes which result in fibroblast-fibrin interactions. Fibrin clot retraction involves calcium-dependent processes such as cell surface receptor binding of fibrinogen and fibrin, cytoskeletal reorganization during spreading and contraction, and secretion of factors involved in fibrin polymerization (56). Indeed, the calcium channel blockers Diltiazem and Verapamil, and the calmodulin (an intracellular calcium binding protein) inhibitor TFP inhibited both FCR activity and fibroblast spreading within fibrin clots (56). The anti-CD9 mAb ALB6 reversed the inhibitory effect of both Diltiazem and Verapamil, but not of TFP. These findings suggest that the CD9 mAb target and those of the two calcium channel blockers may be the same, and that CD9 is involved in the regulation of calcium flux across the plasma membrane.

B. Anti-CD9 mAbs augment neutrophil-endothelial cell interactions

A further role for CD9 in adhesion was evidenced by the ability of anti-CD9 mAbs to stimulate rapid adherence of neutrophils to an endothelial cell monolayer (58). Addition of anti-CD9 mAbs FMC56 and 609-29 induced neutrophil adherence to endothelium by 250% within 20 minutes of mAb addition. As neutrophils are negative for CD9, the anti-CD9 mAb induced adhesion appears to be mediated by the endothelium. Interestingly, anti-CD9 mAb induced neutrophil adhesion is not dependent upon gpIIb/IIIa, the leucocyte integrins (B2 integrins), or protein synthesis, as incubation of anti-CD9 pretreated endothelial cells with mAb M148 (anti-IIb/IIIa), a mAb against B2, or cyclohexanimide, failed to abrogate adhesion. It is likely that anti-CD9 mAb induces an activation event upon the endothelial cell which renders a pre-existing ligand functional. Conversely, anti-CD9 mAbs may induce the surface expression of a ligand for the neutrophil.

E: Anti-CD9 mAbs are potent activators of platelet aggregation

Platelets respond to a wide variety of agonists by undergoing platelet aggregation and activation. These agonists may include monoclonal antibodies binding to their respective cell surface targets. MAb induction of platelet aggregation and activation may occur through a variety of different manners: 1) the binding of the F(ab) portion of IgG to its cell surface receptor may elicit cell activation by promoting a conformational change in the receptor 2) binding of F(ab) portion of IgG may induce local receptor clustering or 3) the interaction of the Fc portion of Ig with cell surface Fc receptors may induce an activation response. The apparent mobility of a receptor within the membrane has been suggested to determine its ability to initiate platelet aggregation. For example, mAbs against the platelet glycoprotein IIb/IIIa, which can cluster on the surface upon binding of mAb, readily aggregates platelets, while a mAb against glycoprotein Ib and IX, which are not mobile, do not promote platelet aggregation (59). A striking property of mAbs within the CD9 cluster is their ability to promote platelet activation and aggregation (60). Modulation of CD9 by

anti-CD9 mAbs initiates release of intracellular calcium, production of TxB₂, phosphoinositide hydrolysis and diacylglycerol formation. Recent studies have suggested that anti-CD9 Ig induced platelet aggregation is mediated entirely by the Fc receptor, and that the CD9 antigen does not participate in signal transduction. Worthington et al (61) showed that F(ab')₂ preparation of anti-CD9 mAb SYB-1 was unable to cause platelet aggregation, but did so if crosslinked with an intact, but not with F(ab')₂ fragments, of a secondary anti-mouse Ig. Furthermore, SYB-1 F(ab')₂ fragments completely blocked binding of intact SYB-1 and ALB6 IgG to platelets, and subsequent mAb induced platelet aggregation. That the Fc receptor and anti-CD9 induced platelet aggregation pathways may be linked was suggested by the observation that the anti-Fc receptor (CD32) mAb IV.3, blocked anti-CD9 mAb induced platelet aggregation, TxB₂ formation, and calcium flux, and by the observations that crosslinking of anti-Fc receptor mAb IV.3 with anti-mouse Ig exhibited similar kinetics of platelet aggregation, calcium flux and cytoplasmic alkalinization, as well as produced equivalent levels of TxB₂, as the anti-CD9 mAb ALB6. These findings imply that anti-CD9 mAb induced platelet aggregation and platelet activation occurs through the Fc receptor pathway.

GIV. CD9 immobilization, and not crosslinking, initiates platelet activation:

It is unclear how the Fc portion of mAb participates in platelet aggregation. However, as indicated above, crosslinking of anti-Fc receptor mAb IV.3 with intact of F(ab')₂ fragment of a secondary anti-mouse Ig invoked a strong activation response, whereas soluble IV.3 alone was insufficient. This suggests that crosslinking Fc receptors to each other, or to other molecules, may signal activation. Indeed, the production of an anti-CD9 mAb, TP 83 (62) capable of activating platelets when used at low concentrations, is a useful tool to investigate the mechanism of anti-CD9 mAb induced platelet activation independent of Fc receptor involvement using a low titre of mAb in an attempt to reduce the involvement of Fc

receptors, Ozaki et al. (63) indicated that platelet activation by anti-CD9 mAb TP 82 proceeds in a two step fashion. The first phase, concomitant to shape change involves phospholipase A2 activation, limited mobilization of arachidonic acid and subsequent limited production of TxA₂. This phase is independent of phospholipase C activation as it does not require elevated levels of calcium or phosphatidic acid formation. The second phase of TP 82 induced platelet activation occurs at the start of platelet aggregation and is characterized by the induction of a massive mobilization of arachidonic acid, which is dependent upon the limited production of TxA₂ from the first phase. Evidence presented by Griffith et al. (64) indicated that CD9 is indeed capable of signal transduction, independent of Fc receptor occupation. In this study, nonstimulatory F(ab)₂ fragments of anti-CD9 mAb, immobilized on a polystyrene latex bead, initiated platelet aggregation in a dose dependent manner. The CD9 activation signal is unique compared to that induced by intact Ig (and hence Fc receptor involvement) as it is entirely dependent upon the secretion of ADP, and a functioning thromboxane pathway, whereas an inhibitor of the cyclooxygenase pathway (aspirin) and the thromboxane receptor antagonist BM13.177, only slightly reduced platelet aggregation induced by the intact Ig.

H. CD9 association with small GTP-binding proteins

The mechanism by which CD9 initiates activation of phospholipase A₂ and subsequent production of arachidonic acid and TxA₂ is unclear. However, the relative importance of membrane located enzymes in the conversion of extracellular signals to intracellular signals is widely accepted. The binding of a ligand, or an activation molecule such as mAb or lectin, to a cell surface protein results in activation of a cell through generation of intracellular signals. The binding of the ligand initiates the production of second messengers by activation, or inactivation of membrane bound enzymes, which can in turn, generate soluble intracellular mediators, or modify proteins. The conversion of information

from the extracellular milieu to the cell interior, and the generation of second messengers may occur through the family of guanine nucleotide binding proteins (GTP-binding proteins) (65, 66). GTP-binding proteins are heterotrimeric proteins ($\alpha\beta\gamma$) derived from a large gene family. There exists roughly 100 different G-protein coupled receptors which include peptide hormones, as well as growth hormones. The G-protein coupled receptors have in common seven membrane spanning domains. Large molecular weight, and small molecular weight G-proteins have been implicated in many aspects of cellular function which include activation, proliferation, differentiation intracellular transport and secretion. Work by Seehafer et al. (67) implicates platelet CD9 in a selective association with two small GTP-binding proteins in the platelet. That CD9 itself did not bind GTP is suggested by the lack of consensus GTP binding site within the CD9 sequence. Lapetina et al. (68) have implicated small GTP-binding proteins in the regulation of phospholipase C (PLC) in the platelet. That CD9 is associated with such regulators suggests that the generation of intracellular signals which occurs upon mAb modulation of CD9 may be catalyzed by small G proteins.

1. Interaction of CD9 with platelet glycoprotein IIb/IIIa:

Many platelet receptors are involved in the process of platelet activation, which involve the binding of soluble mediators such as thrombin and ADP, as well as the recognition of extracellular matrix proteins such as collagen, laminin and fibrinogen. The recognition of these factors results in platelet activation and recognition of components of the subendothelium exposed during vascular injury. Among platelet surface receptors involved in activation and spreading, is included the fibrinogen receptor glycoprotein IIb/IIIa, a member of the integrin family of adhesion receptors. Integrins are a group of receptors shown to be involved in adhesion of cells to other cells, as well as to extracellular matrices. It has now become evident that many integrins require cell activation to expose ligand

binding sites. Circulating platelets do not bind fibrinogen, but upon activation by thrombin, collagen, or other platelet agonists, a binding site for fibrinogen is exposed (70-72). This conformational change may occur through an "inside-out" alteration in the cytoplasmic portion of the protein, such as phosphorylation, or may come about as the result of lateral interactions with other membrane proteins. Interestingly, upon thrombin activation of platelets under conditions which promote cell attachment and spreading, CD9 tends to concentrate in pseudopods and at the periphery of the cell, a distribution similar to GpIIb/IIIa (73). Using the chemical crosslinking agent DSP, Slupsky et al. (74) showed that platelet aggregating doses of anti-CD9 mAbs 50H.19 or ALB₆ promoted association of CD9 with the GpIIb/IIIa complex. CD9 interaction with GpIIb/IIIa could occur directly through anti-CD9 mAb imposed conformational change in CD9 which allows it to bind GpIIb/IIIa, or indirectly through anti-CD9 mAb initiated signal transduction which results in the exposure of a CD9 binding site on GpIIb/IIIa. That anti-CD9 mAb induced CD9:GpIIb/IIIa association occurs in the absence of platelet activation suggests that the association is the direct result of anti-CD9 mAb induced conformational changes in CD9. However, the ability of GpIIb/IIIa to bind fibrinogen upon anti-CD9 mAb binding, requires platelet activation, suggesting that the CD9:GpIIb/IIIa interactions occurs prior to activation signals required to bring about a functional GpIIb/IIIa receptor. CD9 mAbs activate platelet aggregation in the absence of Fc receptor occupation, suggesting that CD9 is capable of initiating intracellular signalling pathways (64). Similarly, mAbs against the β chain GpIIb also activate platelet aggregation, and initiate tyrosine phosphorylation of platelet proteins. The binding of anti-CD9 mAb, which may mimic the natural ligand binding to CD9, may serve to a) promote a conformational change in CD9 which is permissive for the initiation of signal transduction or 2) bring about a conformational change in GpIIb/IIIa, through direct physical interactions with CD9, which may expose ligand binding sites. The combination of the two signalling pathways may be required to exceed the signalling threshold to activate platelets. Interference of the GpIIb/IIIa

signalling event with mAbs against the active receptor, or by peptides against fibrinogen, interferes with, but does not completely reduce, protein tyrosine phosphorylation, an event which occurs upon ligand (fibrinogen) binding, or activation by various agonists. This suggests that ligand binding of GpIIb/IIIa is a necessary, but not sufficient, interaction which occurs during platelet activation. The interaction between GpIIb/IIIa and CD9 may be representative of many complex interactions which occur on the surface of platelets upon exposure to activating agonists, and may serve to regulate platelet binding to subendothelial extracellular matrix components at sites of vascular injury.

II. Adhesion receptors involved in leukocyte function.

A. An introduction to adhesion receptor families

The ability of leukocytes to recognize foreign antigen, receptors on opposing cell surfaces, or extracellular matrix components, is an important element of leukocyte activation and extravasation. Adhesion receptors play a role in a variety of leukocyte functions. Three families of adhesion receptors have been classified which are involved in leukocyte function: 1) the selectins, such as E-selectin, L-selectin, and P-selectin, 2) homing receptor CD44, and 3) the integrin family of adhesion receptors. The homing receptor CD44, while binding to endothelial cells, also recognizes components of the extracellular matrix such as collagen and hyaluronan. The integrins mediate lymphocyte interaction with both components of the extracellular matrix, as well as with other cells. While the remainder of this section is focussed on the discussion of CD44 and integrins, a brief overview on selectins is included to indicate their importance as the primary mediators of leukocyte adhesion and migration during an inflammatory response.

B. Selectins are molecules involved in the initial stages of leukocyte:endothelial cell binding

Leukocyte binding to endothelial cells is required for cells to leave the circulation (extravasation) at sites of inflammation. It is believed that leukocyte extravasation at sites of inflammation is a multi-step process involving multiple adhesion receptors which mediate initial weak interactions with endothelial cells, and subsequent strong interactions which ultimately allow for leukocyte arrest and extravasation. The first step in extravasation is leukocyte rolling along the vessel wall, a process mediated by the selectin family of adhesion molecules. Members of the selectin family, including L-selectin, P-selectin, and E-selectin, are all characterized by a calcium dependent lectin domain and contain C-type lectin domain, EGF domains and a series of complement binding protein domains. E-selectin was discovered on activated endothelium, and binding of E-selectin is considered a first step in the extravasation of blood leukocytes into the site of inflammation (75). P-selectin binding appears to be an initiating step in platelet activation and binding to endothelium (76). L-selectin is important in lymphocyte circulation and is involved in the adhesive events leading to T cell binding to high endothelial venules (HEV) in the peripheral and mesenteric lymph nodes (77). The selectins have been shown to recognize carbohydrate moieties present on the opposing cell. For further details see the following (78).

B. An overview of the CD44 adhesion family

BI. The CD44 adhesion family consists of structural isoforms

The CD44 family of adhesion receptors consists of a collection of structural isoforms which are preferentially expressed by cells of hematopoietic and nonhematopoietic

origin~~er~~. The CD44 isoforms contain distinct peptide sequences generated from differential mRNA splicing, giving rise to an 132 amino acid insertion within a membrane proximal portion of the external domain (79). The alternatively spliced isoform of CD44 is characteristically expressed by epithelial cells. This sequence is minimally conserved among the CD44 isoforms, however, its functional significance is unclear. The major form of CD44 consists of a 37,000 dalton core protein, which is then modified by addition of N- and O-linked carbohydrate and sialic acid (80), or addition of chondroitin sulfate (81). Modifications by glycosylation account in part for the many different forms found in different cell types. Molecular cloning of genes encoding CD44 has provided further information on the structure of this family of molecules. The major structural features of this family are similar for all isoforms of CD44, as evidenced by a high degree of amino acid homology in the external and internal domains. In general, the CD44 gene encodes a protein with an extracellular domain of 248 amino acids followed by a hydrophobic sequence of 21 amino acids, and a 72 amino acid COOH-terminal cytoplasmic tail. The extracellular domain contains seven potential N- and O-linked glycosylation sites near the N-terminal region (82). The cytoplasmic portion contains serines and threonines, but does not contain tyrosine residues. This finding is consistent with reports that CD44 is phosphorylated on serine. Both the N-terminal region and the cytoplasmic portion of CD44 are highly conserved among species (80-90%), suggesting that these sequences may be functionally conserved. Indeed, the N-terminus of the extracellular domain exhibits 30% homology with cartilage link proteins (83, 84). Cartilage link proteins bind extracellular matrix components such as collagen types I and III, as well as hyaluronan (85). The sequence relationship between CD44 and the cartilage proteins suggest a general role for CD44 in proteoglycan or collagen mediated adhesion.

BII. Role of CD44 in cell adhesion

Evidence is accumulating to implicate CD44 in promoting cell-cell adhesion and adhesion to the extracellular matrix. CD44 is involved in lymphocyte trafficking came from the studies which showed that anti-CD44 mAbs could block binding of cells to specialized high endothelial venuels (HEV) (86), which support lymphocyte extravasation to lymph node. CD44 may mediate adhesion through direct ligand binding, or through the activation of other adhesion receptors. To this end, the crosslinking of CD44 with anti-CD44 mAb mediates the adhesion of cells to fibroblasts through the upregulation of CD2 (87). The anti-CD44 mAb H4C4 induced homotypic aggregation of several types of hematopoietic cells requires an intact cytoskeleton, and membrane fluidity (88). Direct evidence linking CD44 expression to adhesion was shown by transfection of murine fibroblasts with CD44 (89). The transfection of the CD44 gene aggregated more readily than did the parental line. The aggregation did not appear to be mediated by interactions of CD44 molecules on opposing cells, as these same transfected cells could form aggregates with the nontransfected parental cell line. This suggests that the CD44 mediated adhesion occurs through CD44 binding of a non-self ligand. It has been reported that a ligand for CD44 is hyaluronan (90, 91). The most convincing line of evidence stems from experiments in which a soluble CD44-immunoglobulin chimera construct bound to hyaluronan, and bound to lesser extent to other glycosaminoglycans (92). The study of Miyake et al. which shows CD44 and HA requirement in the binding of lymphopoietic and myelopoietic precursors to bone marrow stroma provides further evidence for CD44:HA interaction (93).

BIII. CD44 ligand binding requires the cytoplasmic domain

While many CD44⁺ cells bind HA there are examples of CD44⁺ cells which do not bind HA. The variability of CD44:HA interaction may be due in part to ligand specificity exhibited by a particular isoform. Indeed, transfection of the alternatively spliced isoform of CD44 into CD44⁻ B cells did not confer the ability to bind endothelial cells in an HA-

dependent manner (94). This suggests that the expression of CD44 may not be sufficient to confer HA binding ability, or that CD44 may require activation (ie. conversion from a non-HA binding state to an HA binding state) to expose a ligand binding site. The exposure of the binding site may be the result of alterations in the cytoplasmic domain of the molecule (in-side out mechanism), or may be the result of interactions at the extracellular domain (outside in mechanism) either of which could result in a conformational change. Lesley et al. (95) showed that transfection of AKR1 cells with a CD44 containing a truncated cytoplasmic domain did not bind HA, while cells transfected with a nontruncated wild type did bind HA, suggesting that the cytoplasmic domain of CD44 is critical to HA binding. Interestingly, treatment of the truncated CD44 transfectant with an anti-CD44 mAb induced HA binding. This observation suggests that CD44 can be activated to expose a ligand binding site for HA. CD44 activation may come about through a conformational change in the molecule or a change in the distribution of CD44, resulting from coclustering of CD44 or the interaction with other cell surface molecules. Both mechanisms could involve the cytoplasmic domain of CD44, which could alter configuration through interaction with cytoplasmic elements.

BIV. The role of CD44 in lymphocyte activation and differentiation

The ability of CD44 to bind its ligand would not only provide the cell with a means of adherence, but also endow the cell with information regarding its surrounding local environment. The recognition of the surrounding microenvironment (ie. blood vs. tissue), would in part, dictate the cell's response to stimuli. This phenomenon has been observed for integrins VLA-4 and VLA-5, which costimulate T cells upon their recognition of fibronectin. In a similar manner, anti-CD44 mAbs A3D8 and A1G3 markedly enhanced peripheral blood mononuclear cell proliferation induced by anti-CD2 and anti-CD3 mAbs (96). Pretreatment of purified T cell and monocyte populations indicate that anti-CD44

mAb augment monocyte production of IL-1, augment T cell production of IL-2, as well as enhance T:monocyte interactions.

C. An overview of the Integrin family of adhesion receptors

CI. Integrin families

Integrins are heterodimers composed of noncovalently associated α and β subunits expressed by virtually all cell types. In general, integrins are divided into three subfamilies based on α chain usage of a common β chain. The β_3 family, or cytoadhesins, mediate primarily interactions between hematopoietic and nonhematopoietic cells and components of the extracellular matrix such as fibronectin, vitronectin, fibrinogen and vonWillibrand factor. β_2 integrins, or leukocyte adhesion receptors, mediate cell-cell interaction of leukocytes. The β_1 family, or VLA, have been found to mediate both cell-cell as well as cell-extracellular matrix interactions of a variety of cell types. Recent work has added to the list of α and β chains, which now stands at an impressive 13 α , and eight β chains (Table CI-I) (69). A level of complexity is added to the organization of integrin subfamilies in that certain α chains have been recognized to interact with more than one β chain. However, whether the association of an α chain with different (alternate) β chains constitutes novel integrin subfamilies has not yet been determined, as each alternate β chains have so far been described to associate with a particular α chain. With the exception of the discussion on integrin structure, the remainder of this section will focus on the members of the VLA-integrins.

CII. Integrin structure

Both integrin α and β subunits contain a large globular extracellular domain, one transmembrane spanning domain, and a short cytoplasmic domain. The β subunits are very similar, bearing 40-48% amino acid homology, while the α chain amino acid homology is only in the vicinity of 20-30%. Both the α and β chain contain conserved cysteine residues. The α chain contains 14-16 (of 18-24) conserved cyteine residues which are thought to disulfide bond with their nearest neighbour to form short loops which stabilize short-range folding. The β chain contains 56 cyteine residues which are completely conserved, with most cysteins concentrated within four repeating motifs within the C-terminal half of the chain.. Furthermore, it is suggested for β_3 , that the amino terminal portion of the chain is folded into a loop stabilized by disulfide bonds formed between the amino terminal portion and the first cysteine repeating motif. A representative structure of an integrin heterodimer is given in figure CII-1 (97). The cytoplasmic domains of integrins are thought to interact with cytoskeletal proteins. Evidence for this association includes biochemical evidence for the interaction of integrins, or integrin peptides with talin (98. 99) and α -actinin (100). Interestingly, studies using chimeric α subunits indicates that the α chain cytoplasmic domains may dictate various cell behaviour such as motility and adhesion (101)

Some alpha chains (α_3 , α_5 , α_6 , α_V , α_{IIb}) are composed two subunits, formed through posttranslational cleavage, which are joined by a single disulfide bond in the extracellular domain. Another α chain modification is the presence of an 180-200 amino acids insert found in all β_2 integrins, as well as in α_2 . The relevance of these modifications to integrin function is unclear, but it has been suggested that the I-domain may be involved in $\alpha_2\beta_1$ recognition of collagen. The extracellular domain of the α chain contains calcium binding domains which are important in stabilization of the $\alpha\beta$ heterodime and the activation (exposure) of ligand binding sites.

CIII. VLA integrins in cell adhesion and signalling

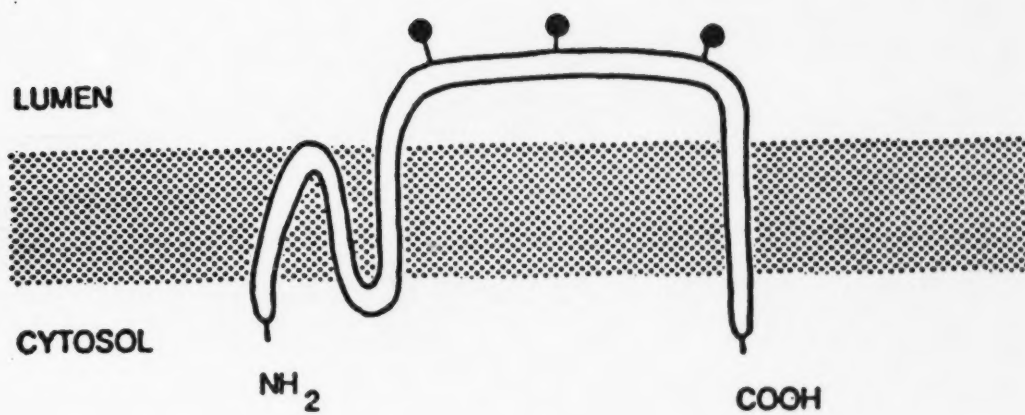
The VLA integrin family, so named due to the late expression of VLA-1 and VLA-2 on activated T lymphocytes, are expressed on a wide variety of hematopoietic cells at all stages of differentiation (102). The majority of ligand for the VLA integrins are components of the ECM, however, cell surface ligands have also been identified, and some VLA integrins can recognize multiple ligands (Table CIII-I). On lymphocytes, VLA-ligand interactions are regulated by cellular activation. Strong adhesion to ECM (103), and similarly to cell surface ligands (104), through VLA receptors can be observed within minutes of activation. The upregulation of VLA upon cell activation not only serves to simply mediate adhesive events, but may also serve to provide information regarding the extracellular environment. Engagement of VLA-4 or VLA-5 by VCAM-1 or FN has been shown to upregulate T lymphocyte proliferation (105, 106) and also play a role in hematopoiesis within the bone marrow (107). Indeed, it has been shown that VLA can transduce intracellular signals (108). The importance of the VLA integrin family in hematopoietic cell development is discussed further in the following references (109-112).

THESIS OBJECTIVES AND OBSERVATIONS:

The association of CD9 with a wide variety of hematopoietic cells suggests that CD9 is involved in a fundamental cellular function. The function displayed by CD9 in fibroblasts clot retraction and in platelet aggregation, and that it is indeed a signal initiating molecule in the platelet, suggests that CD9 may serve to promote adhesion in other cell types. The primary aim of this study was to establish that modulation of CD9 by mAb promotes adhesive events in hematopoietic cells other than platelets. This information would serve to establish a fundamental role for CD9 in cellular adhesion. To answer this question, a pre-B acute lymphoblastic leukemia cell lines were chosen as they express high levels of the CD9 antigen. In chapter I, results indicate that modulation of CD9 by a panel of monoclonal antibodies can induce homotypic aggregation of the pre-B ALL cell lines NALM-6 and HOON. These results suggest a means for homotypic aggregation other than that mediated by anti-leuko-integrin monoclonal antibodies. The mechanism by which anti-CD9 monoclonal antibodies initiate homotypic aggregation of pre-B cells is not understood, however, chapter II suggests that the CD9 and $\beta 1$ signalling pathways may converge to induce homotypic aggregation of the pre-B cell lines. Furthermore, evidence is presented in chapter III to show that mAb modulation of CD9 enhances pre-B cell adhesion to long-term bone marrow stromal cultures, through a mechanism involving the $\beta 1$ integrins VLA-4 and VLA-5 adhesion to fibronectin. These results suggest that CD9 participates in precursor:stromal cell interactions which may impinge upon B cell development. In chapter IV, evidence is presented which shows that modulation of CD9 can indeed induce differentiation of a dendritic cell precursor from peripheral blood. The studies presented within the chapters of this thesis provide evidence which suggests that the CD9 antigen is involved in cellular adhesion of pre-B cells, and that the modulation of CD9 by mAb influences cellular adhesion of a B cell lineage dendritic cell from peripheral blood.

Figure EI. C-1¹

STRUCTURE OF CD9 WITHIN THE PLASMA MEMBRANE



¹ Metzelaar, M. J. 1991. J. Biol. Chem 266:3239.

Properties of the CD9/CD37/CD53/CD63 family members

	Expression	Mol. wt. (kDa)	Polypeptide chain length (amino acid residues)	Comments
CD9 [2] (human)	thrombocytes, pre-B cells	24	227	
CD37 [3,4] (human)	B cells (strongly) other leucocytes (weakly)	40-50	281	intracellularly present in vesicle-like structures [3]; the original sequence [4] was revised and the C-terminus corrected [5]
CD53 [6,7] (human)	leucocytes	32-40	219	CD53 appears to be the best pan-leucocyte marker
CD63/ME491 [8,9] (human)	very broad intracellularly	30-60	228	primarily localized in lysosomal membranes [21]; cell surface expression can be induced by various types of activation and on some tumour cells
TAPA-1 [10,11] (human)	broad	26	236	mAb has reversible antiproliferative effects [10]; associated with several other components including Lew13 [11]; probably not glycosylated
R2 [12] (human)	mRNA strongly upregulated in activated cells	protein undescribed	267	cDNA cloned and sequenced; protein product so far undescribed
CO-029 [13] (human)	some carcinoma cell lines	27-34	237	
rat CD37 [5]		protein undescribed	281	cDNA cloned and sequenced; protein product so far undescribed
OX-44 [4,14] (rat)	leucocytes (except for CD4 ⁺ 8 ⁺ T-lymphocytes)	40-50	219	rat CD53 homologue
Sm23 [15] (<i>Schistosoma mansoni</i>)		23	218	probably not glycosylated
S5.7 (human)	broad			so far unpublished paper by I. Stamenkovic and G. Rovera cited in [7]

¹ from Horejsi, V. et al. 1991. FEBS 288:1.

TABLE CI-1

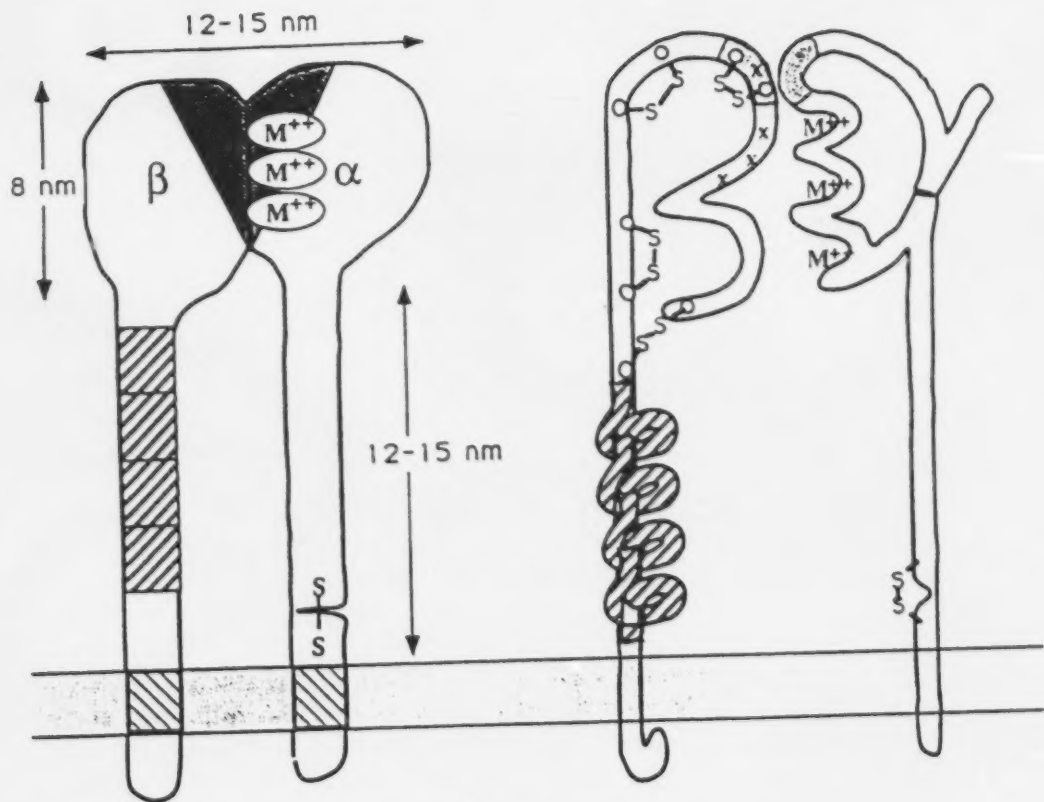
INTEGRIN RECEPTORS AND THEIR LIGANDS

¹	Subunit	Ligand
β_1	α_1	collagen, laminin
	α_2	collagen, laminin
	α_3	fibronectin, collagen, laminin
	α_4	fibronectin, VCAM
	α_5	fibronectin
	α_6	laminin
	α_7	laminin
	α_8	?
	α_v	vitronectin, fibronectin
β_2	α_L	ICAM
	α_M	C3b, fibrinogen, factor X, ICAM
	α_x	fibrinogen, C3b
β_3	α_{IIb}	fibrinogen, fibronectin, von Willebrand factor, vitronectin, thrombospondin
	α_v	vitronectin, fibrinogen, von Willebrand factor, thrombospondin, fibronectin, osteopontin, collagen
β_4	α_6	laminin
β_5	α_v	vitronectin
β_6	α_v	fibronectin
β_7	α_4	fibronectin, VCAM
β_8	α_v	?

¹ from Hynes, R. O. 1992. Cell 69:11.

Figure CII-1¹

INTEGRIN RECEPTOR STRUCTURE



¹ from Hynes, R. O. 1992. Cell 69:11.

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II: ANTI-CD9 MABS INDUCE HOMOTYPIC ADHESION OF PRE-B CELL LINES

A. INTRODUCTION

Cell-adhesion events involved in embryogenesis, histogenesis, and cell migration are determined through receptor recognition of specific extracellular matrix components, and cell surface ligands. Cell-adhesion also plays a critical role in the functioning of the immune system where several families of adhesion molecules have been characterized. These include the lymphocyte homing receptors which define patterns of migration by mediating interaction of lymphocytes with components of high endothelial venules (1), and adhesion molecules such as lymphocyte function-associated antigen-1 (LFA-1) which may contribute to such interaction (2, 3). LFA-1 is a member of the β_2 subfamily of leukocyte integrins, and has been implicated in the mediation of many lymphocyte adhesive functions including cytolytic T cell-target cell interaction (4, 5, 6), and the homotypic aggregation of B, and T lymphoid cells (7, 8). It has been proposed that LFA-1 is not an adhesion initiating antigen, but rather serves to strengthen the adhesion initiated by other molecules (3, 9, 10). LFA-1 binds to one of its ligands, a widely-expressed cell surface glycoprotein ICAM-1 (intercellular adhesion molecule-1) (11), by a mechanism requiring the expenditure of metabolic energy, a functioning cytoskeleton, and the presence of divalent cations (12). Since members of the β_2 integrin sub-family are not found on pre-B cells (13), and LFA-1 is absent from the cell line NALM-6 (14) β_2 integrins are unlikely to mediate the adhesive interactions of these cells. CD9 antigen, on the other hand, is strongly expressed on pre-B cells (15), and we suspected that CD9 might play a role in cellular adhesion since anti-CD9 mAb are recognized initiators of platelet aggregation (16, 17, 18, 19, 20). In this chapter we describe the first evidence that anti-CD9 mAb promote adhesion of nucleated cells, and

that the adhesion has characteristics in common with that induced by LFA- 1, but differs in its requirements for divalent cations.

B. MATERIALS AND METHODS

Monoclonal antibodies: Anti-CD9 mAb's 50H.19 (21), BA-2 (Hybritech Inc., San Diego, CA) and ALB6 (Daymar Laboratories, Toronto, Ont.) are mouse mAbs of the IgG2a, IgG3 and IgG1 isotypes respectively. mAb 9H.1 is an IgG1 reactive with β 2 microglobulin, and in complex with the HLA heavy chain, was produced as described previously (21). mAb BA-1 (Hybritech Inc), and B1, B2, and B4 (Coulter Immunology, Ontario) recognize CD24, CD20, CD21, and CD19 respectively. mAb J5, FMC 71, and WEHI-CAM recognize CD10, CD45R, and ICAM respectively, and were the kind gift of Dr. L.M.Pilarski (Department of Immunology, University of Alberta). mAb BL5 is directed against the β B chain (CD18) of the β 2 integrin sub-family of leukocyte functional antigens, and was purchased from AMAC (Westbrook, ME). MOPC 141 is an IgG2b mouse myeloma protein, and was obtained from Sigma.

Reagents: Ethylenediaminetetraacetic acid (EDTA), ethylene glycol-bis (β aminoethyl ether) N, N, N', N' (EGTA), and cytochalasin B were purchased from Sigma, St. Louis, MO. Bio-Rex 70 ion exchange resin (20-50 mesh, sodium form) was purchased from Bio-Rad Laboratories, Richmond, CA. Arginine-glycine-aspartic acid-serine (RGDS) peptide was purchased from Sigma, and arginine-glycine-glutamic acid-serine (RGES) peptide from Peninsula laboratories (Belmont, CA).

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Cell lines: NALM-6 and HOON are acute lymphoblastic leukemia (ALL) cell lines maintained in our laboratory. NALM-6 was originally obtained from Dr. B. M. Longenecker (Department of Immunology, University of Alberta), and maintained in RPMI 1640 supplemented with 5% FCS, streptomycin and L-glutamine. The pre-B cell lymphoblastoid cell line HOON was kindly provided by Dr. M. Letarte (Department of Immunology, University of Toronto) and maintained in RPMI 1640 supplemented with 10% FCS. Cells used in the aggregation studies were pre-washed 3 times in either phosphate buffered saline (PBS) or Ca^{2+} , Mg^{2+} -free HBSS to deplete divalent cations.

Immunofluorescence analysis: Analysis of surface antigen expression was performed by a combined direct, and indirect staining procedures as previously described (22). Indirect staining was attained by incubating cells with the unconjugated murine antibody for 45 min at 4°C and washing three times before resuspension in biotin conjugated goat anti-mouse (Jackson, U.S.), incubating for 30 min at 4°C, washing twice, and blocking with mouse Ig (Jackson, U.S.) at 0.1 mg/ml at room temperature. Cells were then fluorescently labeled by staining with streptavidine DUOCHROME (Becton-Dickinson, Mountain View, CA) in combination with FITC-, and phycoerythrin-labelled antibodies for 20 min at 4°C, washing three times, and fixing in 1% formalin for flow cytometric analysis. Samples were analysed on a FACScan (Becton-Dickinson) using a single laser. Dead cells were excluded by gating on forward light scatter, and side scatter. All experiments included staining with isotype matched controls. 20,000 cells were analyzed from each sample.

Aggregation assay: The aggregation assay was performed in 1.5 ml. polypropylene tubes (Brinkman Instruments, Westbury, N.Y.). mAb to be tested were added to 2×10^5 cells in 400 μl of assay medium and allowed to bind for 30 minutes at 4°C, before centrifuging the cells at 1,500 $\times g$ for 1 minute, and incubating for a further 60 minutes at 37°C without disruption of the pellet. After incubation, the cells were dispersed by vortex-mixing.

followed by vigorous aspiration with a pasteur pipette, and transferred to a hemocytometer for counting. Non-aggregated cells, and cell-doublets were scored as free cells, and the number of cells which had aggregated determined by subtracting the number of free cells from the total number of cells plated. Cell viability over the assay period was better than 95% as assessed by dye exclusion. The potential modulators of aggregation cytochalasin B, RGDS, and RGEs peptides were preincubated with the cells for 30 min before addition of mAb.

Studies determining cation dependency were carried out in Ca^{2+} -, Mg^{2+} -free HBSS supplemented with Ca^{2+} -, Mg^{2+} -free FCS. CaCl_2 and MgCl_2 were added back to the assay medium at the concentrations indicated in the text. Since mAb ALB₆ produced a low but significant amount of aggregation in the absence of divalent cations the % NALM-6 aggregation shown in Fig.5 represents the aggregation induced by the mAb in the presence of the divalent cations after subtraction of the % aggregation induced by the mAb in $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free HBSS. The studies determining the effect of temperature on aggregation required that the assay medium and cells be equilibrated at 4°C for one hour before the start of assay and maintained at the indicated temperature throughout the course of the aggregation assay.

C. RESULTS

1. NALM-6 and HOON cell phenotype

A phenotypic analysis of the pre-B cell lines NALM-6 and HOON was undertaken to confirm their status as pre-B cells, and determine their expression of β_2 integrins. Three parameter immunofluorescence analysis demonstrated that more than 90% of the cells of both cell lines expressed the pre-B cell markers CD9, CD19, CD10, and CD24 (Fig.1a), whereas less than 3% of the cells expressed CD18 (Fig.1b), I-CAM, CD20, positive for

HLA DR, and weakly positive for CD45RA (Fig. 1) compatible with a pre-B cell phenotype (23). HOON cells were found to express a lower level of CD45RA than NALM-6 cells suggesting that they represent an earlier stage of differentiation. The cells completely lacked the low molecular weight isoform of CD45 (p180) which is present on activated B cells (22) further confirming their pre-B cell phenotype. The non-lineage marker LFA-3 (CD58) was also strongly expressed on both lines (Fig.1). Therefore, with the exception of 45RA, the two cell lines possess an almost identical phenotype suggesting a close lineage relationship.

2. Anti-CD9 mAb induces aggregation

Single cell suspensions of NALM-6 cells exposed to a platelet-aggregating concentration (10ug/ml) (20) of mAb 50H.19 had formed large homotypic aggregates containing more than 100 cells when observed at 48hr (Fig.2A) whereas control cultures without addition of mAb did not show evidence of aggregation (Fig.2B). Aggregate formation was observable as early as 6 hr and became maximal by 48 hr. The aggregates were very stable being resistant to mechanical disruption by vortex-mixing, or vigorous pipetting. Since aggregate formation took place slowly, presumably through random contacts, it was time-consuming and difficult to quantitate. We therefore developed a short term assay to quantitate anti-CD9 mAb-induced homotypic aggregation. The assay, described in Materials and Methods, reduced the time necessary to demonstrate aggregation to 60 min by bringing the cells into close contact through centrifugation, and greatly reduced experimental variability. The assay was first employed to define the dose-response relationships of the aggregation induced by mAb ALB6, and 50H.19. mAb ALB6 was found to be the more potent antibody promoting the aggregation of NALM-6 cells at concentrations as low as 60 ng/ml, and aggregating a maximum of 80-85% of the cells at concentrations of 4 ug/ml, and above (Fig.3A). In contrast mAb 50H.19 required a concentration of 2.5 ug/ml to become

minimally effective, and induced aggregation of only 35-45% of the cells at the optimal concentration of 10 ug/ml (Fig. 3B). The dose-response relationships of the mAb titred against HOON cells were very similar to those obtained with NALM-6 (data not shown). The anti-CD9 mAb were unable to induce aggregation of the CD9 negative B cell line Raji even at concentrations of 20 ug/ml (data not shown).

3. Specificity of mAb-induced adhesion

The specificity of the aggregation response to anti-CD9 mAb was illustrated by the fact that neither mAb 9H.1 (anti-HLA class-I), nor BA-1 (anti-CD24) were able to induce NALM-6 cell aggregation (Fig.4A) or HOON cell aggregation (Fig.4B) even though they labeled the cell lines as effectively as anti-CD9 mAb (data not shown, and Fig. 1). mAb 9H.1 has the same isotype as mAb BA-2, and therefore controls for possible involvement of Fc receptor dependent pathways of activation. mAb MOPC-141 which does not bind to the pre-B cell lines was employed as a negative control, and was also without effect on aggregation (Fig.4).

4. Anti-CD9 mAb induced aggregation requires divalent cations

CD9-induced aggregation of both NALM-6 cells, and HOON was totally inhibited by 4 mM EDTA indicating a requirement for divalent cations. We employed the aggregation assay to investigate those requirements on NALM-6 cells, and found that the aggregation induced by mAb ALB₆, and BA-2 had an absolute requirement for Ca²⁺, but not for Mg²⁺ (Fig.5) indicating that Ca²⁺ is both necessary, and sufficient to produce aggregation. However, the response was less than that obtained in physiologically balanced medium. The Ca²⁺ concentration was not limiting since increasing the concentration of Ca²⁺ from 1mM to 4mM did not produce further aggregation. However,

1mM Mg^{2+} in the presence of saturating levels of Ca^{2+} considerably enhanced aggregation (Fig.5) indicating that Mg^{2+} -dependent adhesion is also under the control of CD9. The less potent mAb 50H.19 failed to promote aggregation in the presence of either Ca^{2+} , or Mg^{2+} but did so in the presence of both cations further indicating that CD9 requires both Ca^{2+} , and Mg^{2+} to become maximally effective. The control mAb 9H.1 induced a very low level of aggregation which was not enhanced by the addition of divalent cations (Fig.5).

5. Anti-CD9 adhesion requires a functioning, cytoskeleton, the expenditure of metabolic energy, and a physiological temperature

LFA-1 induced cellular adhesion to target cells, or to ligands in artificial membranes is an active process requiring a functioning cytoskeleton, the expenditure of metabolic energy, and showing temperature-dependence (12, 24). Since CD9 also induces strong cell-cell adhesion we investigated whether it had similar requirements. We found that anti-CD9 mAb induced aggregation is also completely inhibited by cytochalasin B (Fig.6), an inhibitor of microfilament assembly (25), added prior to the initiation of the assay, and by sodium azide (10 mM) (data not shown) indicating a requirement for a functional cytoskeleton, and for the expenditure of metabolic energy. Further evidence that CD9 is inducing an active process was provided by the finding that aggregation was temperature dependent being reduced to less than 10% of the value obtained at 37°C if the assay were conducted at 4°C (Fig. 6). The inhibition was, however, reversed by returning the cells to 37°C demonstrating that the viability of the cells had not been compromised. In contrast once aggregation had been established at 37°C it could not be reversed by reducing the temperature to 4°C indicating that although cell adhesion was dependent upon the expenditure of metabolic activity for its initiation it did not require metabolic energy for its continuation.

6. Modulation of anti-CD9 induced aggregation by RGD -containing peptides

Cell-matrix interactions mediated by integrins including the fibrinogen receptor, vitronectin receptor, and fibronectin receptor are inhibitable by RGD-containing peptides (26). In contrast the cell-cell adhesion mediated by LFA- 1 does not appear to require the recognition of an RGD sequence (12). It was therefore of interest to determine whether CD9-induced aggregation could be blocked by an RGD-containing peptide. The adhesion promoted by an agonistic dose of mAb ALB6 was not inhibited by a concentration of RGDS peptide (Fig.7) sufficient to abrogate the thrombin-induced aggregation of platelets (data not shown) indicating that anti-CD9 mAb-induced adhesion is not mediated via receptor structures requiring recognition of an RGD sequence.

D. DISCUSSION

In this report we describe the generation of strong homotypic aggregation of nucleated cells following stimulation with anti-CD9 mAb. This is a novel observation which extends the adhesion-regulatory activities of CD9 beyond the platelet, and suggests that the promotion of cellular adhesion may be a general function of CD9. The mechanisms of cell-cell adhesion mediated by LFA-1, and induced by CD9 have much in common. Both have a requirement for the expenditure of metabolic energy, maintenance of physiologic temperature, and a functioning cytoskeleton, and both result in the generation of strong levels of cell-cell adhesion which are not reversible by mechanical means. These properties distinguish them from the type of cell-cell adhesion determined solely by receptor-ligand

interaction such as LFA-2 (CD2) with LFA-3 (CD58) which can proceed at 4°C (24, 27), and suggest that they regulate a co-ordinated cellular mechanism requiring structural organization of the cytoskeleton. However, despite these similarities LFA-1 mediated, and CD9-induced aggregation show striking differences in their specific requirements for divalent cations. Whereas LFA-1 mediated homotypic cell aggregation as well as CTL target binding requires the presence of Mg^{2+} but not Ca^{2+} (7, 9, 28) we found that the converse is true for CD9-induced aggregation. The ability of Ca^{2+} ions to support CD9 induced adhesion implicates a Ca^{2+} -dependent receptor-ligand interaction. However, the ability of Mg^{2+} to enhance anti-CD9 mAb-induced aggregation in the presence of saturating levels of Ca^{2+} suggests that anti-CD9 mAb may also initiate Mg^{2+} -dependent adhesion events which require Ca^{2+} to become effective. Lymphocyte adhesion under the regulation of LFA-1 rarely proceeds spontaneously (7), but more usually requires prior cellular activation by antibody (8), antigen (10, 28), or phorbol ester (9, 12) suggesting that activation of protein kinase C, which normally requires an increase in intracellular Ca^{2+} , may be a key element in that process.

A role for intracellular Ca^{2+} in the activation of integrins to a functional state is further supported by the ability of agonists which induce transients in cytosolic Ca^{2+} to expose a function-associated antigenic neo-determinant on Mac-1, another member of the $\beta 2$ sub-family of integrins (29). Since the cation requirements of LFA-1 adhesion have been determined only on activated cells (12) any Ca^{2+} -dependency of the activating step would not have been apparent. Anti-CD9 mAb induced aggregation, on the other hand, does not require prior activation of the cells, or platelets, and might initiate a divalent cation-dependent intracellular activating signal prerequisite to divalent cation-dependent adhesion. It is therefore of interest that anti-CD9 mAb are reported to antagonize the inhibitory effects of Ca^{2+} -channel blockers on fibroblast interaction with extracellular matrix components (30), and to effect changes in Ca^{2+} -uptake by platelet membrane vesicles (31), properties

which suggest that CD9 may regulate the passage of Ca^{2+} across the plasma membrane. Neither HOON, nor NALM-6 cells aggregate spontaneously, or during the centrifugation and incubation steps of the aggregation assay indicating that exposure of the cells to anti-CD9 mAb induces a unique condition necessary for adhesion.

CD9 is a 22 kDa glycoprotein and may be too small to provide the binding capability required of a major adhesion structure. The ability of all three anti-CD9 mAb to effect aggregation demonstrates that they did not block adhesion, but rather initiated an adhesive process which must be effected by other molecules. It has been reported that anti-CD9 mAb promote fibrinogen binding to the platelet fibrinogen receptor through the generation of intracellular signals (19). It is therefore of interest that we have recently shown using chemical cross-linkers that anti-CD9 mAb also promote a close physical association between CD9, and the platelet fibrinogen receptor suggesting a role for CD9 in the function of that receptor complex (32). Our failure to find evidence for the involvement of RGD-dependent integrins in CD9-induced aggregation of NALM-6 cells by attempting to inhibit their adhesion with RGD-containing peptides suggests that RGD sites are not involved in the processes under the control of CD9. This finding does not, however, preclude the involvement of integrins since neither LFA-1-mediated adhesion (12), nor VLA-6 mediated interaction with its ligand laminin (33) are sensitive to inhibition by RGD peptides.

The expression of CD9 on the ALL cell lines NALM-6, and HOON suggests that nontransformed pre-B cells may also be under the regulation of CD9, a possibility supported by the finding that the 3% of bone marrow cells which are brightly staining for CD9 also comprise about 50% of the terminal deoxynucleotidyl transferase positive progenitor lymphocytes in that organ (15). Since CD9 is also expressed on some mantle zone, and germinal center cells of B origin, but not on mature plasma cells or their precursors (34) it may serve to promote cell-cell interactions of importance to the early

Figure 1a

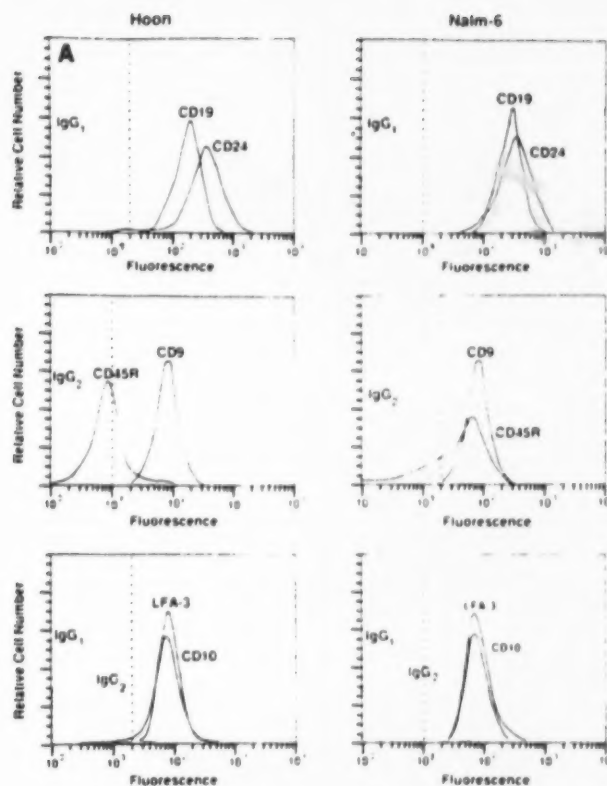


Figure 1. Phenotype analysis of the ALL cell lines HOON, and NALM-6. Expression of B cell markers, CD45R, LFA-3 (Fig.1a) and CD18 (Fig.1b) was analyzed by staining with mAb as described in Materials, and Methods. Cells were analyzed by immunofluorescence, and flow cytometry. Histograms are shown together with isotype matched controls. Fluorescence intensity is on a logarithmic scale.

Figure 1b

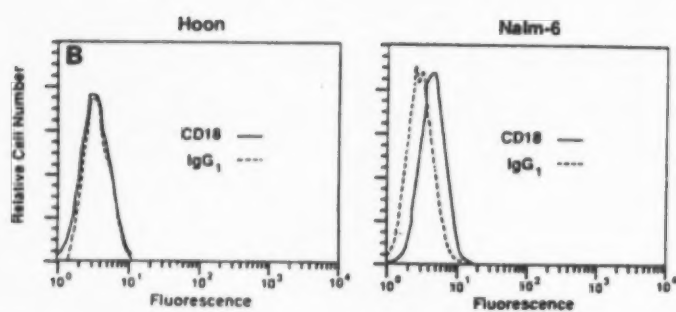


Figure 2

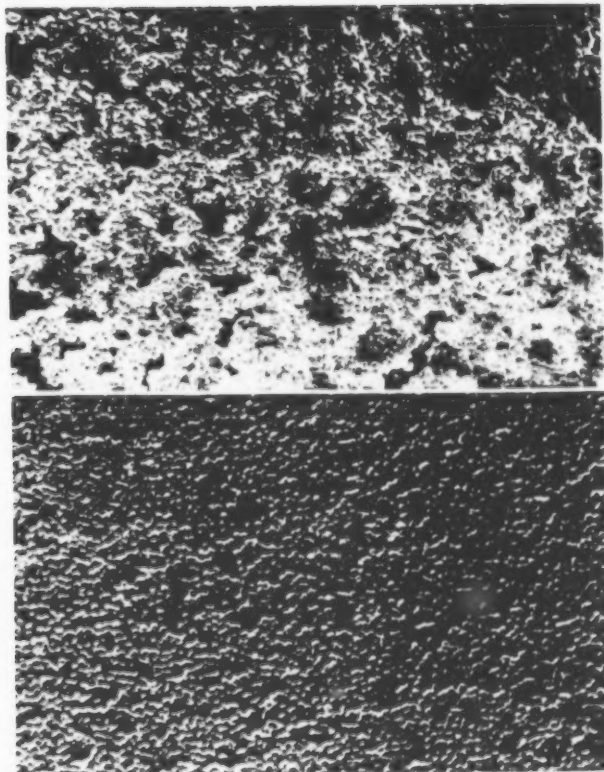


Figure 2. Photomicrographs of anti-CD9 mAb-induced aggregation. 1×10^5 NALM-6 cells were mechanically dispersed and plated as a single cell suspension in RPMI 1640 supplemented with penicillin/streptomycin and 5% FCS, and incubated for 48 hr in either the presence (A), or absence (B) of the anti-CD9 mAb 50H.19 (10ug/ml). The photomicrographs are at the same magnification.

Figure 3

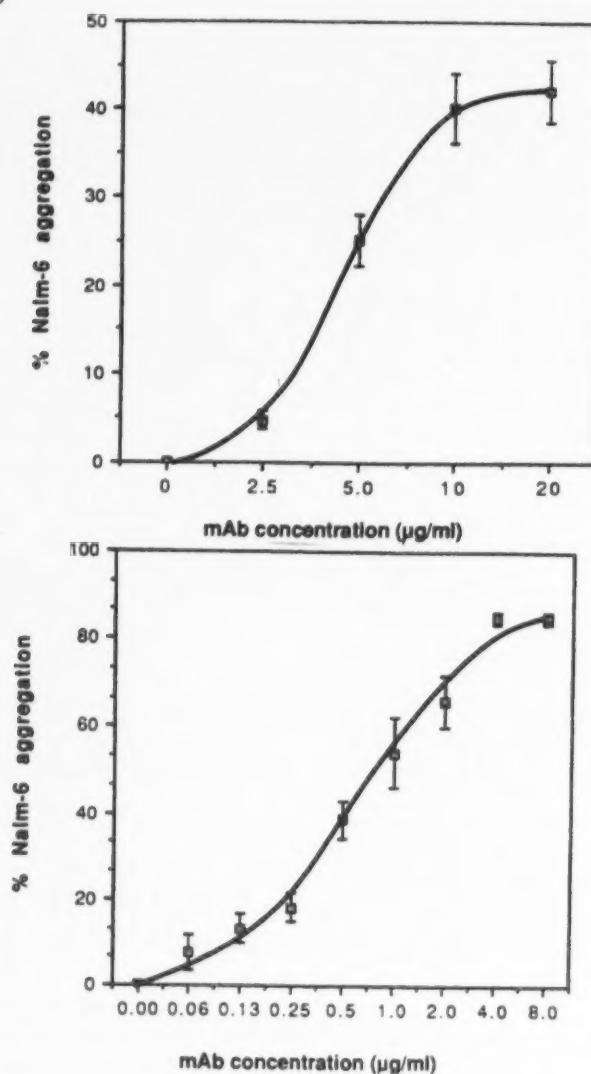


Figure 3. Dose-dependency of anti-CD9 mAb-induced aggregation of NALM-6 cells. Varying concentrations of the anti-CD9 mAbs ALB₆ (A) or 50H. 19 (B) were added at the initiation of the aggregation assay described in Materials and Methods. The results are the mean of 6 experiments. Error bars indicate one standard deviation.

Figure 4

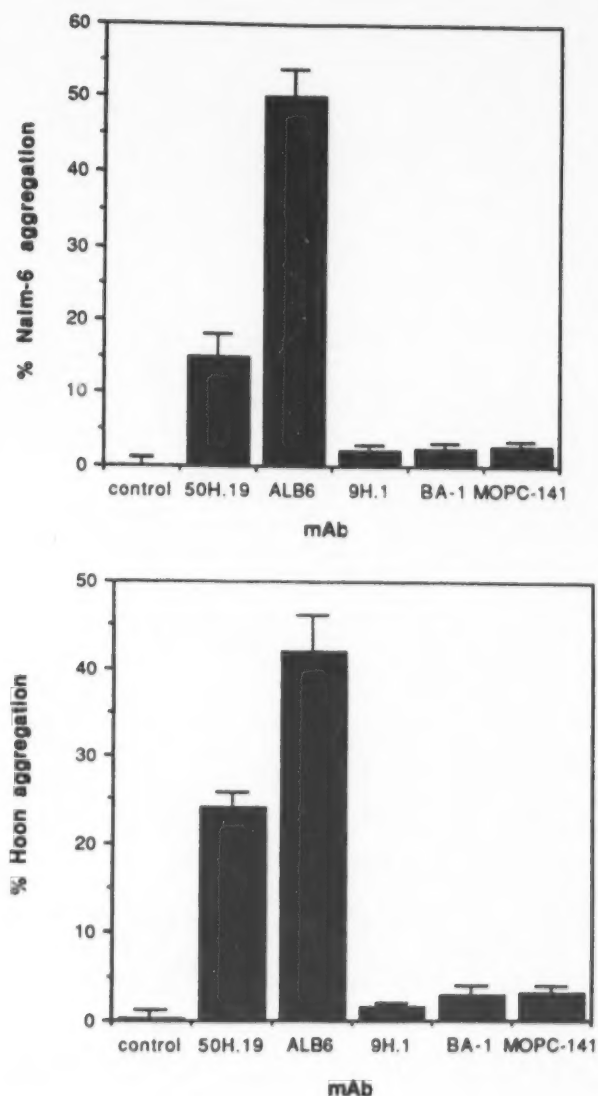


Figure 4. Specificity of anti-CD9 mAb-induced aggregation. Single cell suspensions of A. NALM-6 cells, and B. HOON cells were exposed to mAb 50H.19 (5.0 ug/ml), ALB6 (0-5 ug/ml), 9H.1 (10 ug/ml), BA- 1 (0.5 ug/ml), or MOPC- 141 (10 ug/ml) in the aggregation assay (Materials, and Methods). The control is without antibody. The results are representative of six experiments. Error bars indicate one standard deviation.

Figure 5

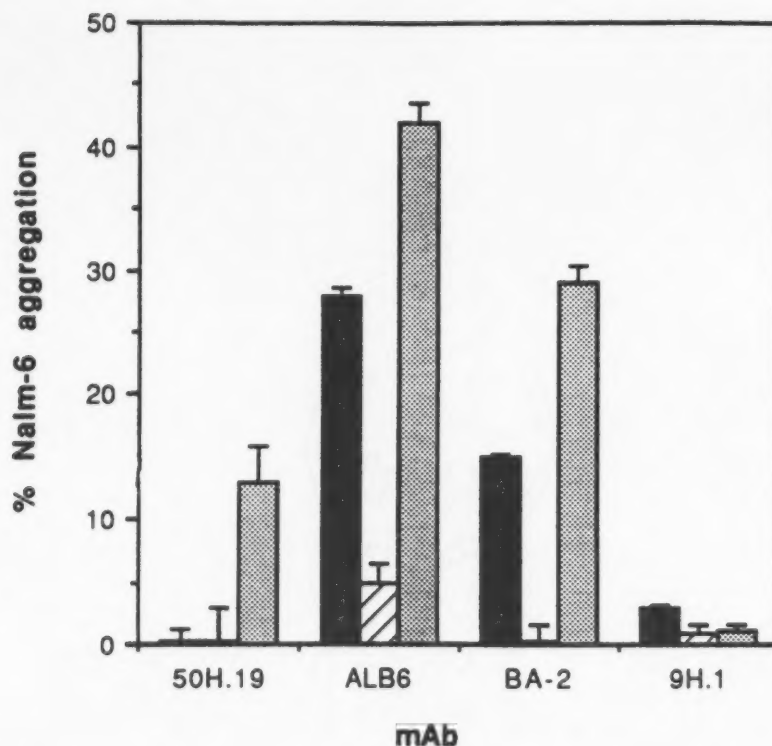


Figure 5. Divalent cation requirements of anti-CD9 mAb-induced aggregation. NALM-6 cells were washed once in $\text{Ca}^{2+}/\text{Mg}^{2+}$ free HBSS and supplemented with 4mM EGTA and twice with $\text{Ca}^{2+}/\text{Mg}^{2+}$ free HBSS supplemented with 2mM EDTA. Cells were resuspended in $\text{Ca}^{2+}/\text{Mg}^{2+}$ free HBSS before addition of 1mM CaCl_2 (dark shaded bars), 1mM MgCl_2 (hatched bars), or a combination of the two (light shaded bars). The aggregation assay was performed as described in Materials and Methods in the presence of mAb 50H.19 (10 $\mu\text{g}/\text{ml}$), ALB₆ (2 $\mu\text{g}/\text{ml}$), or BA-2 (2 $\mu\text{g}/\text{ml}$). The results are representative of 4 experiments. Error bars indicate one standard deviation.

Figure 6

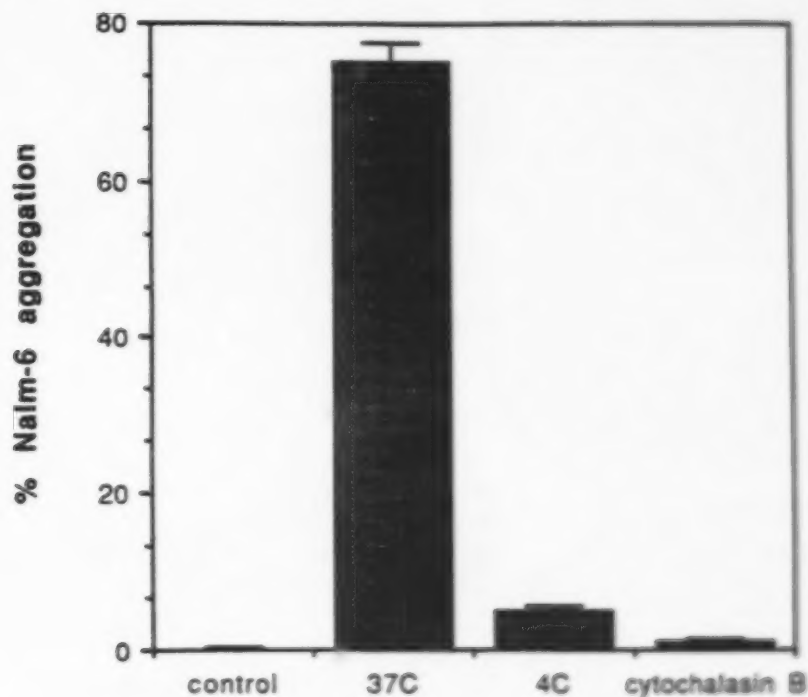


Figure 6. Effect of temperature and cytochalasin B on anti-CD9 mAb-induced aggregation. mAb ALB6 (4 ug/ml)-induced aggregation of NALM-6 cells was determined at 4°C or 37°C. NALM-6 cells were maintained at 37°C, or pre-equilibrated to 4°C prior to assay. Cytochalasin B (20uM) in DMSO was added just prior to initiation of the aggregation assay and maintained throughout. Controls with DMSO alone did not differ from those without (not shown). The results are representative of three experiments. Error bars indicate one standard deviation.

Figure 7

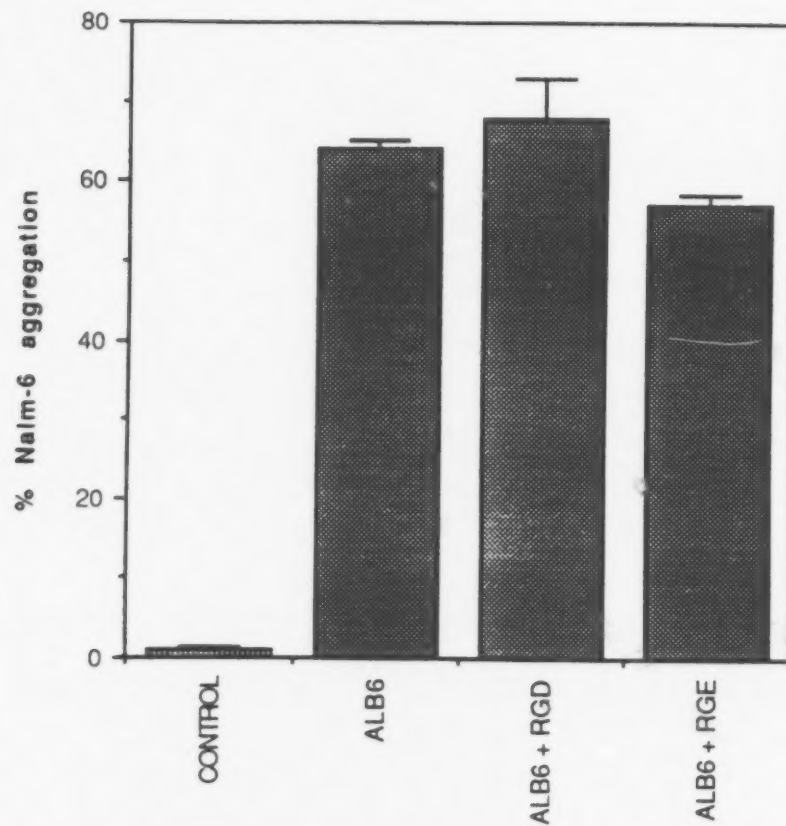


Figure 7. Effect of RGD peptide on mAb ALB₆-induced aggregation of NALM-6 cells. The cells were pretreated with 1mM RGDS peptide, or 1mM RGE peptide in HBSS prior to addition of mAb ALB₆ (1 μ g/ml) and assay. The results are representative of three experiments. Error bars indicate one standard deviation.

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III: HOMOTYPIC AGGREGATION OF PRE-B CELL LINES BY ANTIBODIES TO VLA INTEGRINS CORRELATES WITH THE EXPRESSION OF CD9¹

A. INTRODUCTION

Adhesion plays an important role in the function of the immune system [1, 2] where expression of adhesion receptors at different stages of lymphocyte maturation determines accessory cell interactions, and trafficking of lymphocytes between immune compartments [1, 3, 4, 5]. Leukemogenesis and progression involve interaction of malignant cells with stromal and hemopoietic elements within the bone marrow [2, 6, 7] and with the components of the vessel wall. Defining the molecular basis by which leukemic cells regulate their adhesive interactions is therefore fundamental to an understanding of the leukemic process.

Integrins constitute a major class of adhesion proteins composed of diverse non-covalent heterodimers (α , β) grouped into several subfamilies on the basis of their α and β subunit association. VLAs are a subfamily of integrins first recognised for their role in the function of lymphocytes [8]. VLA integrins are characterized by association of the β_1 subunit (CD29) with any of the α^1 to α^6 subunits [5]. In a previous chapter we described several molecules at the surface of the acute lymphoblastic leukemia (ALL) cell line HOON [9]. The HOON cell line bears the characteristic markers of a pre-B lymphocyte namely CD19, CD10/CALLA, HLA-DR and no surface Ig [9, 10, 11]. 85% of cases of childhood leukemia are of pre-B phenotype and since normal pre-B human cells are not readily obtained in culture systems, these cells, and cell lines constitute the most available source of human pre-B cells. In this work we have used mAb to investigate the expression and function of cell surface proteins continuously, or discontinuously expressed on a series of

¹ from Letarte, M., et al. 1992 *Leukemia* 7:93.

five human pre-B cell lines established from relapse cases of childhood acute lymphoblastic leukemia.

A heterodimer consisting of two non-covalently linked subunits of Mr 125,000 and 87,000 was previously isolated from the HOON line by affinity to mAb 44H6-IgG [9]. Cellular distribution studies revealed that the antigen was primarily restricted to leukemic cells, and to hemopoietic and lymphoid tissues [9, 12]. The distribution and subunit structure of 44H6 antigen suggested a relationship to VLA-4 integrin [5, 8, 13] which is distinguished from other integrins by the endogenous, and variable cleavage of its a subunit into fragments of Mr 80,000 and 70,000 [14, 15]. By analysis of the proteins recognized by mAb 44H6 in comparison to mAb B-5G10, known to react specifically with the VLA-4 integrin [14], we establish here that 44H6 mAb is reactive with VLA-4, and show VLA-4 to be the major VLA integrin of pre-B leukemic cells.

Recent observations indicate that mAb reactive with certain epitopes on VLA-4, can induce the homotypic aggregation of B and T lymphocyte tumor cell lines, a process requiring divalent cations, the expenditure of metabolic energy, and an intact cytoskeleton [16, 17, 18]. This report is, to our knowledge, the first account of homotypic aggregation regulated by anti-integrin mAb in pre-B cells. We demonstrate that mAb 44H6 (anti- α -4 integrin), and mAb 4B4 (anti- β 1 integrin) induce strong homotypic aggregation of pre-B cells, and that the extent of aggregation correlates with the level of the ALL marker CD9 on the cell lines.

CD9 is a leukemia and lymphopoietic cell surface antigen expressed by developing, but not mature B lymphocytes, which is a marker for pre-B cell leukemia [19], and is present as differentially glycosylated isoforms of 22 and 24 kDa [20, 21]. Approximately 90% of non-T leukemias express the antigen which is also found on about 50% of acute myeloid

and chronic lymphoid leukemias. Although the precise role of CD9 is not understood the protein was recently cloned [22] and assigned to a newly described family of multiply-inserted transmembrane proteins which are proposed to function as receptor-gated ion channels [23]. The family includes the B cell antigen CD37 [24], the pan-leukocyte marker CD53 [25], and the melanoma stage-specific antigen ME491 (CD63) [26]. A striking property of mAb directed against CD9 is the ability to activate platelets [21, 27, 28] suggesting a role in the initiation of signals directing adhesion. We recently reported that anti-CD9 mAb could induce not only platelet aggregation, but also the homotypic aggregation of pre-B cells suggesting that CD9 performs a fundamental role in adhesive processes [29]. Although the precise function of CD9 in the induction of platelet aggregation is unknown, we and others have reported evidence for both a physical and functional association between CD9 and the $\beta 3$ integrin GPIIb/IIIa [30, 31]. In this paper we present novel evidence suggesting a functional association between CD9, and the ability of VLA proteins to induce pre-B cell adhesion.

B. MATERIALS AND METHODS

Cell lines. The pre-B ALL cell lines HOON, NALM-6 and A1 have been described previously [10, 32, 33]. The G2 and C1 lines were derived from relapse samples of pre-B ALL cases by Dr. M. H. Freedman (Division of Hematology/Oncology, Hospital for Sick Children, Toronto, Canada). The U-937 pro-monocytic cell line [34] and the pre-B cell ALL lines were maintained in RPMI 1640 supplemented with 10% fetal bovine serum.

Antibodies. The mAb 44H6 was prepared and purified as described [9]; it is of IgG1 isotype. The mAb B-5G10 directed at the VLA-4 ($\alpha 4$) subunit was obtained as a partially purified ascites from Dr. M.E. Hemler, Dana-Farber Institute, Boston, MA [8]. The mAb 4B4 (anti- $\beta 1$ integrin) and mAb B4 (anti-CD19) were purchased from Coulter, Hialeah,

FL, while mAb P1D6 (anti-VLA-5, α^5 subunit), P1E6 (anti-VLA-2, α^2 subunit) and P1B5 (anti VLA-3, α^3 subunit) were purchased from Telios, San Diego, CA. TS1/18 and TS1/22 are IgG1 hybridomas reacting with human LFA-1 α and β subunits; they were kindly provided by Dr T. A. Springer, The Center for Blood Research, Boston, MA. The ALB6 IgG1 (anti-CD9) and 84H10 IgG1 (anti-ICAM-1) were purchased from Bio/Can Scientific Inc, Mississauga, Ontario. mAb 50H.19 (anti-CD9) is an IgG2a [22] and was obtained from Dr. B.M. Longenecker, Department of Immunology, University of Alberta, Edmonton, ALTA. The mAb 44G4 to human endoglin is an IgG1 [9, 35, 36]; mAb 21w4, of IgG1 isotype, reacts with HLA-DR [37]. The mAb 44D10 and 50B4, of IgG1 and IgG2b isotype respectively, are reactive with CD44 [9, 38]. A rabbit polyclonal antiserum reactive with b1 integrins was purchased from Telios. The rat anti-VLA-6 mAb GoH3, of IgG2a isotype was obtained from Bio/Can Scientific. mAb 197 to the Fc γ RI (CD64) of IgG2a isotype and mAb IV.3 to the Fc γ RII of the IgG2b isotype were obtained from Medarex Inc., West Lebanon, NH. Purified control IgG1 and IgG2b were obtained from Coulter, Hialeah, FL.

Flow cytometry. Cells (1×10^6) were stained with saturating amounts of mAb and FITC-conjugated F(ab')₂-goat anti-mouse IgG (Tago Laboratories Burlingame, CA). Samples were run on the Epics Profile Analyzer (Coulter Electronics, Hialeah, FL). Linearity was established with immunobeads and conditions standardized daily by adjusting the voltage in the fluorescence channel such that Immunoclone #3 beads gave a mean log₁₀ fluorescence intensity of 5.25. Thus all histograms are directly comparable and relative fluorescence units are linear with respect to log₁₀ fluorescence values. Isotype controls are run in every experiment and the position of these controls indicated in all histograms. All experiments involving IgG2a were done in the presence of 5 ml human AB serum. This is particularly critical for cells which express the Fc γ RI such as U-937.

¹²⁵I-surface labeling, immunoprecipitation, and SDS-PAGE: Cells were washed three times with PBS and lysed with extraction buffer (2% Triton X-100, 10 mM Tris-HCl pH 7.4, 5 mg/ml BSA, 2mM PMSF, and 10 mg/ml each of leupeptin, pepstatin, and antipain). Lysates were precleared with Protein A Sepharose CL-4B (Pharmacia), immunoprecipitated with mAb attached to Protein A Sepharose by incubation for 12 h at 4°C with rotation, and the immunoprecipitated proteins released from the beads by extensive washing in extraction buffer containing 0.5 M NaCl were analyzed by SDS-PAGE and autoradiography.

Peptide maps: ¹²⁵I-labeled proteins were immunoprecipitated from Triton X-100 lysates and separated by SDS-PAGE on 7% gels. Slices containing protein bands were overlaid on 12.5% polyacrylamide gels, digested with 100 mg/ml Staphylococcus aureus V8 protease (Boehringer Mannheim, Laval, Quebec) during SDS-PAGE, and the peptides identified by autoradiography.

Homotypic aggregation assay. Experiments were performed in triplicate in 96 well Linbro tissue culture plates with 1.5×10^5 cells/well essentially as described [16 and chapter 1]. Harvested cells were washed several times in RPMI 1640 medium without fetal bovine serum and resuspended in RPMI medium supplemented with 20 mM HEPES and 0.8% BSA. In most experiments, fetal bovine serum at 1% was added. Only purified IgG fractions of antibodies were used in the assays as the presence of variable amounts of fetal bovine serum in culture supernatants rendered the assessment of the contribution of the serum to aggregation uninterpretable. The aggregation was scored after 16-24 h, at 37°C in a humidified incubator, 5% CO₂. All photomicrographs were taken at a magnification of 100x using a Leitz Diavert microscope equipped with a Wild Photoautomat MPS45 System and using Kodak Tech Pan film.

C. RESULTS

1. mAb 44H6 is reactive with VLA-4 integrin

The reactivity of mAb 44H6 was compared to that of mAb B-5G10, a defined anti-VLA-4 antibody [8]. The level of staining observed with these two antibodies was indistinguishable with each of the pre-B leukemic lines tested. To confirm that these two antibodies were indeed recognizing the same molecules, immunoprecipitation experiments were performed. The mAb 44H6 and B-5G10 immunoprecipitated from radiolabeled NALM-6 cells similar polypeptides: major bands of 150 kDa and 130 kDa (reduced) and of 145 kDa and 115 kDa (non-reduced) with minor bands of 80 kDa and 67 kDa (reduced) and 78 kDa and 63 kDa (non-reduced) (Fig. 1A). The major polypeptides correspond to the α and β subunits of VLA-4, and the minor bands to the non-disulfide linked cleavage products of the α chain [13]. Limited proteolysis of the subunits with *Staphylococcus aureus* V8 protease produced identical peptide maps from the two proteins (Fig. 1B) confirming that the two antibodies recognized the same protein.

2. Distribution of VLA integrins on pre-B leukemic cells

Staining with an antibody reactive with the β_1 subunit of integrins (mAb 4B4) gives an indication of the total number of classically defined VLA molecules available at the cell surface [5]. With all cell lines tested but G2, the mean fluorescence intensity observed with 4B4 was higher than observed with any of the α chain specific antibodies (Fig. 2). The highest reactivity with antibodies to the distinct α subunits was seen with anti-VLA-4. With HOON, NALM-6, A1 and C1 lines the ratios of the mean fluorescence intensities observed with anti- α^4 and anti- β_1 were 0.6, 0.8, 0.6 and 0.5, respectively, while with G2 line, this ratio was 1.5. These results first indicate that VLA-4 is the major VLA molecule on all of the lines tested, and secondly suggest that the G2 line might contain an additional complex of α^4 associated with a distinct β chain, such as VLA-4 alt ($\alpha^4\beta_p$) [39, 40, 41].

All cell lines were negative with antibodies to α^2 and α^3 and three of the lines showed reactivity with anti- α^5 . The highest levels were observed with the C1 line (ratio of 0.4 to β^1) while NALM-6 and HOON expressed equivalent levels (ratio of 0.2 to β^1). The laminin receptor VLA-6 was found predominantly on the A1 cell line, but also at low, but detectable levels on most of the other lines. Thus reactivity with anti- α^4 and α^5 antibodies appear to account for most of the VLA molecules present on pre-B ALL cells.

3. Homotypic aggregation of pre-B cell lines with antibodies to VLA-4 and VLA-5

The mAb 44H6 was tested for its ability to induce homotypic aggregation of pre-B cell lines based on the observation that several anti- α^4 antibodies have this property on certain T, B and myelomonocytic human lines [16, 17, 18, 42]. As seen in Fig. 3, mAb 44H6 can induce significant aggregation of HOON, NALM-6 and G2 cells while only a very low level of aggregation was observed with C1 cells and none with A1 cells (Fig. 3). The aggregation was blocked by the addition of the blocking mAb B-5G10 which is directed at VLA-4; mAb B-5G10 did not induce aggregation when present by itself (Fig. 3 and Table I). The anti- β^1 mAb 4B4 induced strong aggregation of HOON and NALM-6 cells which was only partially inhibited by mAb B-5G10. Aggregation of the G2 cell line seems to be more variable, and not stronger with anti- β^1 than with the 44H6 anti- α^4 antibody. The C1 cells gave a very low level of aggregation with 4B4 while the A1 cells were negative. These results should be contrasted with the histograms of Fig. 2 which reveal high levels of β^1 and α^4 on all five pre-B cell lines. Thus aggregation is not strictly correlated with the presence of VLA-4 integrin.

Three of the lines express similar levels of α^5 (Fig. 2). Aggregation of these 3 lines (HOON, NALM-6 and C1) was observed at levels of 3, 2 and 0-1 respectively (Fig. 3 and

Table I). Thus the presence of VLA-5 is not sufficient to explain the level of aggregation induced via that ligand

The aggregation events induced by 4B4, 44H6 and P1D6 mAb were inhibited with 0.1% sodium azide. Although aggregation could be observed in the absence of fetal calf serum, the kinetics of aggregation and the size of the clusters formed were increased in the presence of 1% fetal calf serum without significantly altering the background observed in the presence of purified IgG1 or IgG2b, or with mAb which did not induce significant aggregation (Table I).

4. Differential expression of LFA-1, ICAM-1, CD44 and CD9 on pre-B leukemic cell lines

In an attempt to elucidate other cell surface molecules of pre-B cells implicated in the process of homotypic aggregation, we analyzed the distribution of antigens previously shown to be involved in leukocyte aggregation. The HOON, NALM-6, A1 and C1 cells are LFA-1 negative while the G2 line expresses this antigen (Fig. 4), thus not correlating at all with the ability of these cells to aggregate. The ICAM-1 antigen, an LFA-1 ligand, is expressed on the HOON, A1 and C1 lines. Consequently none of the cell lines express both LFA-1 and ICAM-1 which would be a necessary condition if the homotypic aggregation of the pre-B cell lines were mediated via interaction of this receptor-ligand complex.

Antibodies to CD44 have been shown to induce homotypic aggregation of several types of hemopoietic cells [43]. Furthermore the expression of CD44 on pre-B ALL cells is high and variable [44]. Fig. 4 reveals that all cell lines but NALM-6 express CD44. The highest level is observed on the A1 line which does not aggregate at all with any antibody tested (Table I). The NALM-6 cell despite the absence of CD44 is capable of aggregation.

Consequently no correlation was observed between the presence of CD44 and the ability of pre-B cells to form aggregates.

CD19 is a characteristic marker of leukemic cells of B lineage whose expression appears very early in pre-B cell differentiation and is relatively constant on pre-B ALL cells obtained from patients [11, 44]. Fig. 4 reveals that indeed the five lines tested do express similar levels of CD19 and that variation in their ability to undergo VLA-induced aggregation cannot be explained by variations in the levels of the protein.

CD9 is an antigen present on pre-B ALL [19, 20] which has been implicated in the homotypic aggregation of platelets and pre-B cells [21, 27, 28, 29]. Fig. 4 indicates that CD9 is present on the pre-B cell lines in the following order: HOON > NALM-6 > G2 > C1 > A1 cells. High levels are observed on HOON and NALM-6, both cell types capable of undergoing high levels of VLA-mediated aggregation. The G2 line shows low levels of CD9 and is capable of intermediate levels of aggregation. The C1 line expresses very low levels of CD9 and shows minimum aggregation. The A1 line is CD9 negative, and does not aggregate at all. Thus there appears to be a correlation between VLA-induced aggregation and the level of CD9 present on the pre-B cells not observed with the other markers tested.

5. Homotypic aggregation of pre-B cells observed with anti-CD9, anti-CD19 but not with anti-CD44.

The ability of anti-CD9, CD19, CD44 (and of other antibodies to cell surface components of pre-B ALL) to independently induce homotypic aggregation was investigated. The ALB6 antibody (anti-CD9) can induce aggregation of HOON, NALM-6 and G2, but not of A1 or C1 cells. Table I summarizes the results of several experiments with these 3 cell

lines. Combinations of anti-VLA-4 and anti-CD9 mAb neither potentiated nor inhibited one another suggesting that these antibodies may activate the same signal transduction pathway.

Table I illustrates that the anti-CD19 antibody, B4, can induce aggregation of HOON cells, but not of NALM-6 cells and, to a variable degree, of G2 cells. The addition of anti-CD19 to anti-VLA-4, or anti- β 1 neither increased nor inhibited the aggregation induced by these antibodies. Similarly, the addition of anti-CD19 antibody did not affect the aggregation induced by anti-CD9.

CD44 an 85 kDa glycoprotein implicated in homing of lymphocytes to high endothelial venules [45] has been implicated in the aggregation of certain hemopoietic lines by a mechanism which is resistant to sodium azide [43]. We did not obtain aggregation of pre-B cell lines with anti-CD44 mAb (Table I).

The anti-HLA-DR antibody 21w4, reactive with the 5 pre-B cell lines, did not induce aggregation, nor did the 44G4 anti-endoglin antibody reactive with both HOON and NALM-6 cells [35, 36, 44]. Thus binding to a cell surface antigen of pre-B cells via an IgG1 or IgG2 antibody is not sufficient to induce aggregation indicating that Fc receptor mediated mechanisms are not involved.

6. Fc receptors are not implicated in the process of homotypic aggregation

Despite the observation that $F(ab')_2$ fragments of anti-VLA-4 could mediate homotypic aggregation at ng levels [16], residual uncleaved IgG might account for this effect. In the current study we assessed the presence of Fc receptors on pre-B cell lines. The Fc γ RII of human cells which binds mouse IgG1 and IgG2b with relative specificity was found at low levels on the pre-B cell lines (Fig. 5). The percentage of positive cells were 26, 36, 54, 12,

and 15 for HOON, NALM-6, G2, A1 and C1 respectively. The highest amount was therefore observed on G2 cells which exhibit, at best, only intermediate levels of aggregation suggesting that Fc mediated mechanisms of activation are not involved. Fc γ RI (which binds specifically to monomeric mouse IgG2a) was not observed on the pre-B cells (Fig. 5). The U-937 cell line which expresses both receptors was included as a positive control [46, 47].

When a 10-20 fold excess of mAb IV.3, directed at the Fc γ RII, was present during the aggregation of HOON or NALM-6 cells, induced by anti-b1, anti-VLA-4, or anti-CD9 no inhibitory effect was observed (Table II). F(ab')₂ fragments (10mg/ml mAb) of the anti-CD9 mAb 50H.19 from a preparation which was incapable of inducing platelet aggregation [54] induced full aggregation of pre-B cells further supporting the Fc-independence of mAb-induced signalling in these cells (Table II). These findings indicate that the Fc γ RII is not involved in the mAb induced homotypic aggregation of pre-B cells.

D. DISCUSSION

In this study we establish that VLA-4 and VLA-5 are the major integrins expressed on a series of pre-B leukemic cell lines. Since certain of the pre-B leukemic cell lines undergo vigorous homotypic adhesion in response to mAb directed against VLA- α 4, the β 1 integrin subunit, or to a lesser degree VLA- α 5, pre-B cell adhesion appears subject to regulation by integrins as previously described for T and B lymphoblastoid cell lines [16, 17, 18, 42]. However, in contrast to T and B cell lines the homotypic aggregation of pre-B cells induced by anti-integrin antibodies appears to critically depend upon factors which are variably expressed by the cell lines.

The potency with which mAb against selected epitopes of VLA integrins can induce the aggregation of pre-B cells suggests a mechanism by which the interaction of physiologically appropriate ligands with VLA-integrins can modulate the state of cellular

adhesion. As suggested for VLA-induced aggregation of human T and B cells [17, 42], and for the induction of LFA-1/I-CAM-1 interaction [48], the antibodies may induce changes in the avidity, or in the stereoselectivity of the integrin for as yet unidentified ligand(s) constitutively expressed on the cell surface [17]. By binding to critical epitopes such as divalent cation binding sites on VLA- α 4 [42] and GPIIb [49] mAb may effect changes in the conformation of the receptor facilitating interaction with other ligands. The conformational lability of integrins is supported by evidence for activation-dependent neo-epitopes on the β 2 integrins LFA-1 [50], Mac-1 [51], and the β 3 integrin GPIIb/IIIa [52]. Recently topographically, and functionally distinct epitopes on the VLA- α 4 chain have been identified using mAb [42], exemplified in this study by mAb44H6 and B5G10, which counter-regulate adhesive interactions suggesting a physiological mechanism by which VLA-4-dependent aggregation might be effected through interaction with different ligands. Our results therefore confirm the importance of VLA-4 as an initiator of lymphocyte aggregation and establish that the induction of homotypic aggregation of pre-B cell lines by anti- α 4, α 5 or β 1 subunit antibodies is not simply related to their level of expression. We had previously reported that mAb against CD9 potently stimulate homotypic aggregation of the pre-B cell lines NALM-6 and HOON by a mechanism requiring cytoskeletal assembly, the expenditure of metabolic energy, and the presence of Ca^{2+} [29, chapter 1], properties which are also characteristic of anti-VLA-4 induced homotypic aggregation of T and B cells [16, 17, 18]. In the present study the inability to either potentiate, or inhibit the level of aggregation by admixing anti-CD9 and anti-VLA-4 mAb indicates that CD9 and VLA proteins may be coupled to a common effector mechanism. We previously demonstrated a physical association between CD9 and β 3 integrins in human platelets under conditions in which anti-CD9 mAb effect granule release and aggregation indicating that CD9 may play a role in β 3 integrin signalling [31]. The evidence presented here suggests that CD9 also plays a role in effecting an adhesive phenotype following stimulation of members of the β 1

subfamily. However, it is not clear whether CD9 is required to transduce the integrin signal, or to modulate it.

The role of CD9 as a signalling molecule in human platelets is questioned by the Fc-dependency of anti-CD9 mAb induced activation [53]. We recently demonstrated that F(ab')₂ fragments of anti-CD9 mAb which are non-stimulatory in solution become agonistic if presented on the surface of latex beads [54] indicating that CD9 does indeed play a role in signal-initiation. The unique requirement in platelets for solid phase presentation of the stimulus indicates that CD9-signalling may contribute to mechanisms for evaluating mechanical stress. The ability of integrins to form connections between the cytoskeleton, and the extracellular matrix would be congruent with a role for these molecules in the transduction of mechanical stresses associated with cellular adhesion. The induction of platelet aggregation by anti-VLA- α 4 and anti-CD29 mAb in the presence of mAb IV.3 indicates that the small numbers of Fc γ IIR we detected on pre-B cells are not required for VLA-triggered adhesion. The ability of soluble anti-CD9 mAb to also effect pre-B cell aggregation in the absence of Fc engagement, supports an independent signalling role for CD9, and indicates that pre-B cells respond to simple crosslinking of CD9 without the need for immobilisation. Why platelets and pre-B cells should differ so markedly in their signalling requirements has yet to be characterised. If CD9 does direct a common pathway in platelets and pre-B cells this finding would indicate that platelets have a much higher threshold for mechanical stimulation.

A physiological role for homotypic aggregation in B cell development has yet to be clearly identified. The ability of pre-B cells to form homotypic aggregates may serve as a regulatory mechanism to assemble cells at similar stages of lymphoid differentiation within the cellularly diverse bone marrow environment. Alternatively, since mAb directed at integrins are reported to induce not only homotypic, but also heterotypic aggregation, the mechanisms involved in homotypic adhesion may be relevant to adhesive interactions between different cell types [17]. Developing B cells depend upon intimate contact with

stromal cells in order to respond to mitogenic signals [3, 6, 7], and adhere to stroma through a mechanism involving VLA-4 [7]. The selective expression of CD9 at early stages of B cell maturation imply a role for this molecule in directing the activities of pre-B cells within the bone-marrow micro-environment. The absence of $\beta 2$ integrins on the majority of pre-B cell lines contrasts with the widespread expression of LFA-1 on B and T cell lines and indicates that LFA-1 performs adhesive functions more relevant to cellular interactions within the peripheral lymphoid organs and vasculature than within the bone marrow [7]. The absence of coincident expression of ICAM-1 and LFA-1 on pre-B cell lines is compatible with our inability to promote, or enhance homotypic aggregation of pre-B cell lines by exposure to phorbol esters. Although B cell adhesion was originally described to take place by LFA-1/ICAM interaction in response to PMA [55] mAb engagement of CD19, CD20, CD39, CD40, and HLA-DR can also induce homotypic adhesion in B lymphocytes by two mechanisms; one which involves LFA-1, and another which is LFA-1 independent and is probably not integrin-mediated since it does not require external cations [56]. The finding that neither mAb stimulation of CD19, nor the addition of PMA could induce the homotypic aggregation of two pre-B cell lines in those studies [56] coupled with similar findings presented here indicate that pre-B cells utilise alternative pathways to effect cellular adhesion. Pre-B cells appear to have a restricted repertoire of adhesion mechanisms at their disposal operating through either CD9 [29], or as suggested here through VLA-integrins. The correlation established between the expression of CD9 and the functional competence of VLA suggests that these pathways are closely inter-related, and that CD9 may determine the integrin-mediated adhesive phenotype of pre-B leukemic cells. CD9 expression may therefore critically affect the genesis and dissemination of that disease. Mice with combined severe immunodeficiency (*scid* mice) are permissive hosts for human leukemic cells, but largely of cells derived from patients in relapse [57] suggesting that the murine bone-marrow micro-environment favors the growth of more aggressive human leukemic cells which are less dependent upon host interaction. It is of interest therefore that

the pre-B cell lines A1 and G2, and cells from patient R3 from which the line C1 was derived, proliferated extensively in the bone-marrow of *scid* mice, and disseminated to several tissues, including brain, leading to the eventual death of the host [57,58]. These cell lines, which are poorly responsive to the induction of antibody-mediated aggregation, are therefore able to grow and disseminate *in-vivo*. The *scid* mouse model offers the opportunity to investigate whether the level of CD9 expression on leukemic cells determines their potential to proliferate and disseminate *in-vivo*.

Figure 1

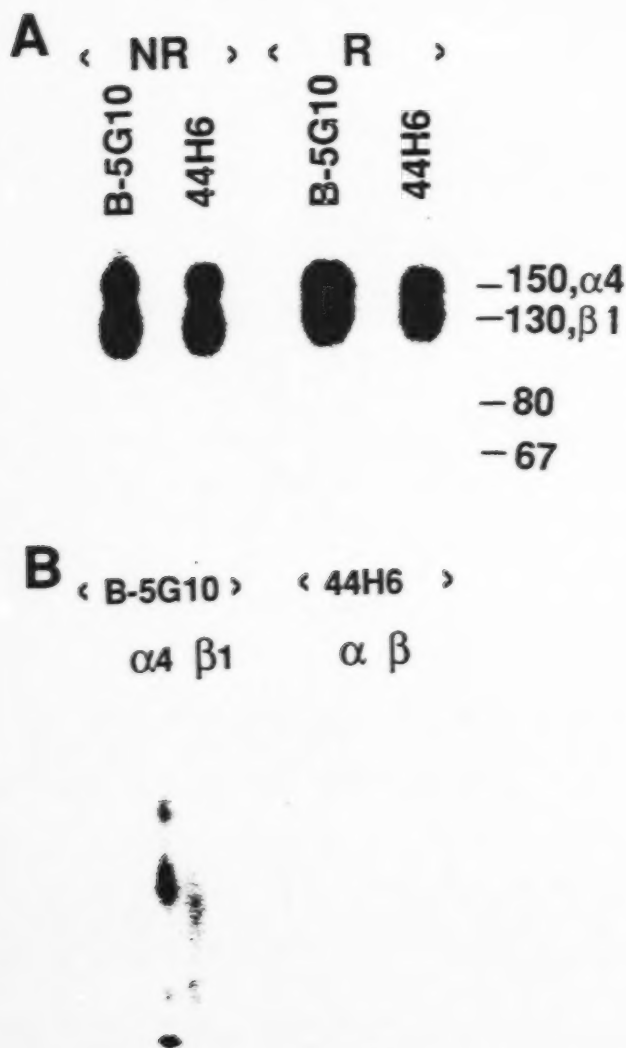


Figure 1: Immunoprecipitation and peptide map analysis confirm that 44H6 and B-5G10 epitopes are expressed on the same molecules.

A. ^{125}I -labeled NALM-6 cells were lysed with Triton X-100, and immunoprecipitated by the anti-VLA-4 mAb B-5G10 and 44H6 and analyzed by SDS-PAGE on 7% gels under non-reducing (NR) and reducing (R) conditions. Proteins of identical mobility were immunoprecipitated by both mAb corresponding to $\alpha 4$ chain, $\beta 1$ chain and the 80 and 67 kDa cleavage products of $\alpha 4$.

1B. Gel slices (reduced) containing the α and β subunits of the proteins immunoprecipitated by mAb B-5G10 and 44H6 were overlaid onto 12.5% gels and digested with *Staphylococcus aureus* V8 protease during electrophoresis. The peptide maps derived from the α and β chains immunoprecipitated by mAb B-5G10 ($\alpha 4$, $\beta 1$) and 44H6 (α , β) are identical.

Figure 2

VLA Integrins on Pre-B Leukemic Cells

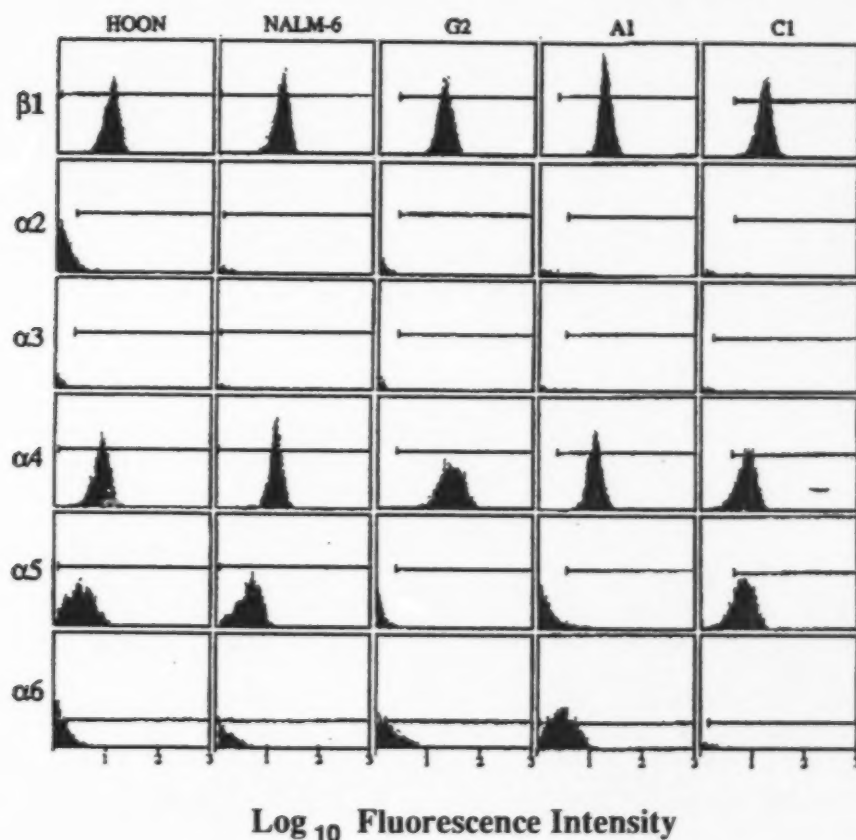


Figure 2: Distribution of VLA integrins on pre-B leukemic cell lines. Samples (1×10^6 cells) of the leukemic pre-B cell lines indicated were incubated with saturating levels of mAb 4B4 (anti- $\beta 1$), P1E6 (anti- $\alpha 2$), P1B5 (anti- $\alpha 3$), 44H6 (anti- $\alpha 4$), P1D6 (anti- $\alpha 5$), and GoH3 (anti- $\alpha 6$) followed by FITC-F(ab')₂-goat anti-mouse IgG and analyzed using the Coulter Epics Profile. With anti- $\beta 1$ >99% cells were labeled with mean fluorescence values of 2.4, 3.8, 4.5, 4.7 and 3.8 respectively. With anti- $\alpha 2$ and $\alpha 3$, < 1% cells were labeled except for 5% of HOON cells reacting with anti- $\alpha 2$. With anti- $\alpha 4$, >99% cells were reactive and the mean fluorescence values were 1.5, 3.0, 7.7, 2.6 and 1.7. With anti- $\alpha 5$, 91%, 96%, 1%, 3% and 80% positive cells were observed with mean fluorescence values of 0.5, 0.7, 0.9, 0.9 and 1.4 respectively. With anti- $\alpha 6$ 22%, 24%, 28%, 91% and 3% positive cells were detected with mean fluorescence values of 0.2-0.4.

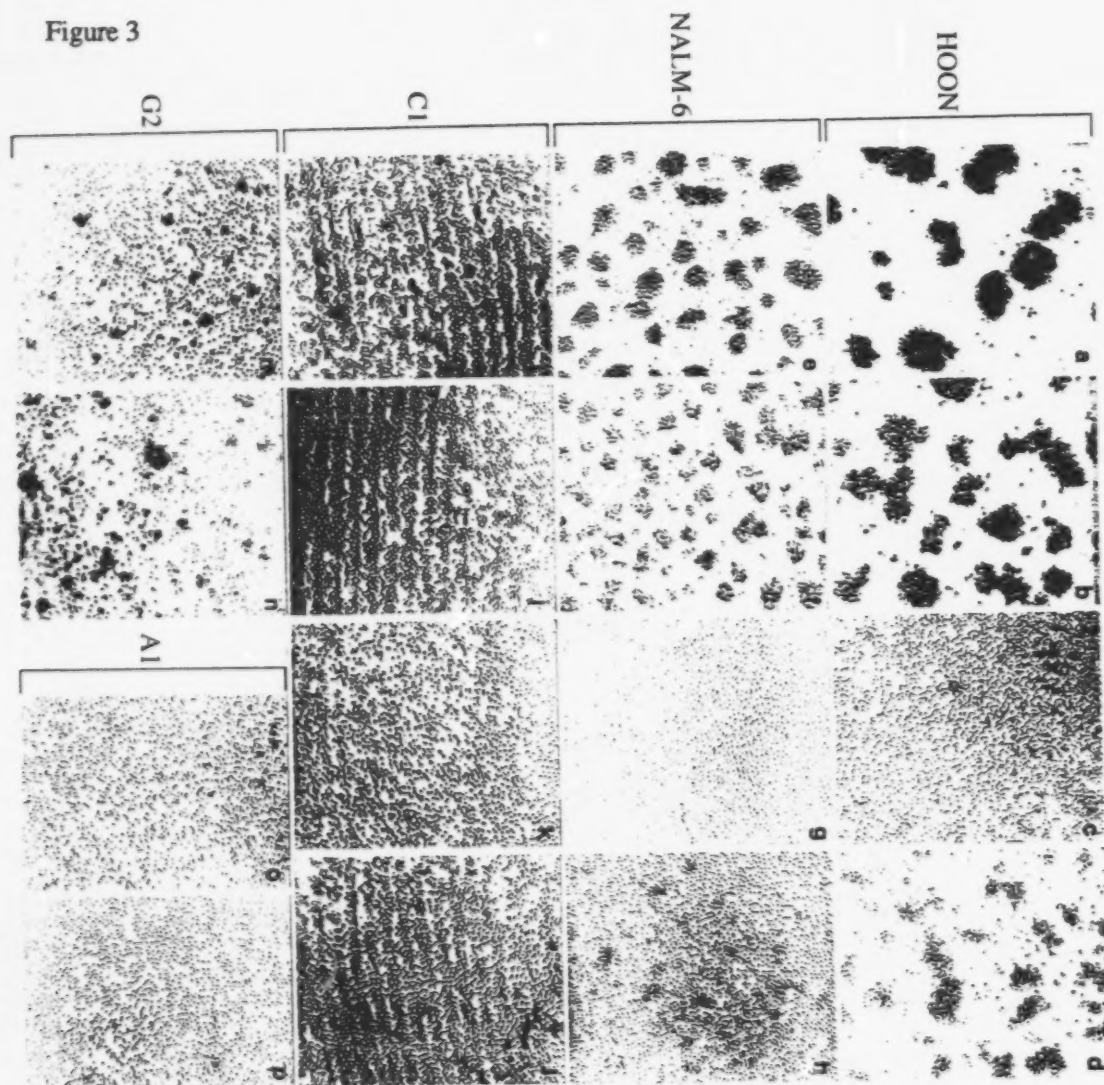


Figure 3: Homotypic aggregation of pre-B leukemic cells via VLA integrins. The five pre-B leukemic lines indicated were tested for their ability to aggregate when incubated with mAb 4B4 (anti β 1), in a, e, i, m and o; with mAb 44H6 (anti- α ⁴) in b, f, j, n and p; with a combination of mAb 44H6 and B-5G10 (both anti- α ⁴) in c and g; with IgG1 control antibody in panel k; and with mAb P1D6 (anti-VLA-5) in d, h and l. Purified IgG were used except for B-5G10 (partially purified ascites) and P1D6 (ascites) at the optimal concentrations indicated in Table 1. All assays were done in medium containing 1% fetal bovine serum and scored at 24 h.

Differential Phenotype of Pre-B Leukemic Cells Tested for Homotypic Aggregation

Figure 4

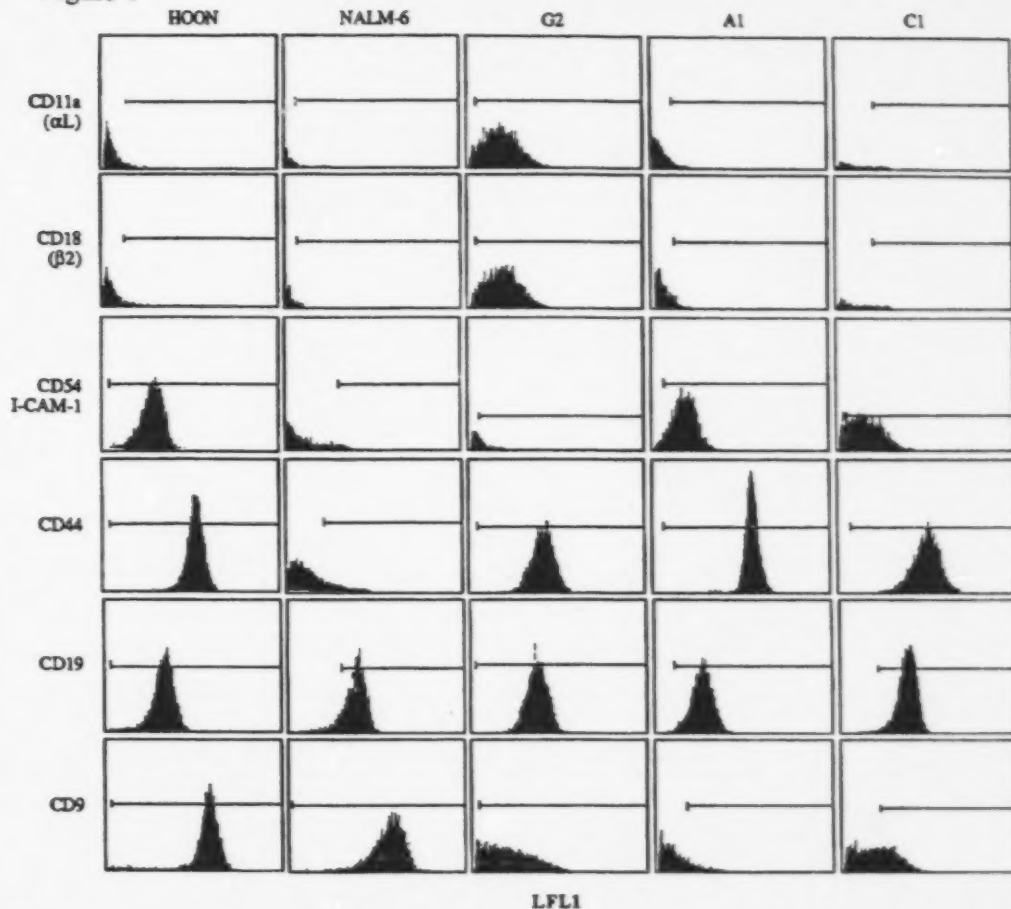


Figure 4: Surface expression on pre-B leukemic cells of molecules other than VLA and potentially implicated in homotypic aggregation. The five pre-B cell lines were tested by flow cytometry with mAb to the antigens indicated: TS1/18, TS1/22, 84H10, 50B4, B4 and ALB6 respectively. Only the G2 line expressed significant levels of the CD11a-CD18 integrin (90% positive cells, mean fluorescence 0.6). The CD54 antigen is present on HOON cells (99%, mean fluorescence 1.3), A1 cells (85%, mean fluorescence 0.8) and C1 cells (69%, mean fluorescence 0.5). The NALM-6 cells are CD44 negative while >99% of HOON, G2, A1 and C1 cells express CD44 with mean fluorescence values of 12.2, 4.7, 18.9 and 9.4. All cells express CD19 with mean fluorescence values of 2.1, 3.7, 3.0, 1.3 and 3.0, respectively. The CD9 expression is in the following order, HOON (99%, mean fluorescence 18.2) NALM-6 (99%, mean fluorescence 17.1), G2 (68%, mean fluorescence 0.7), C1 (27%, mean fluorescence 1.3) and A1 (7%, mean fluorescence 0.9).

Expression of Fcγ RI and Fcγ RII on pre-B Leukemic Cells

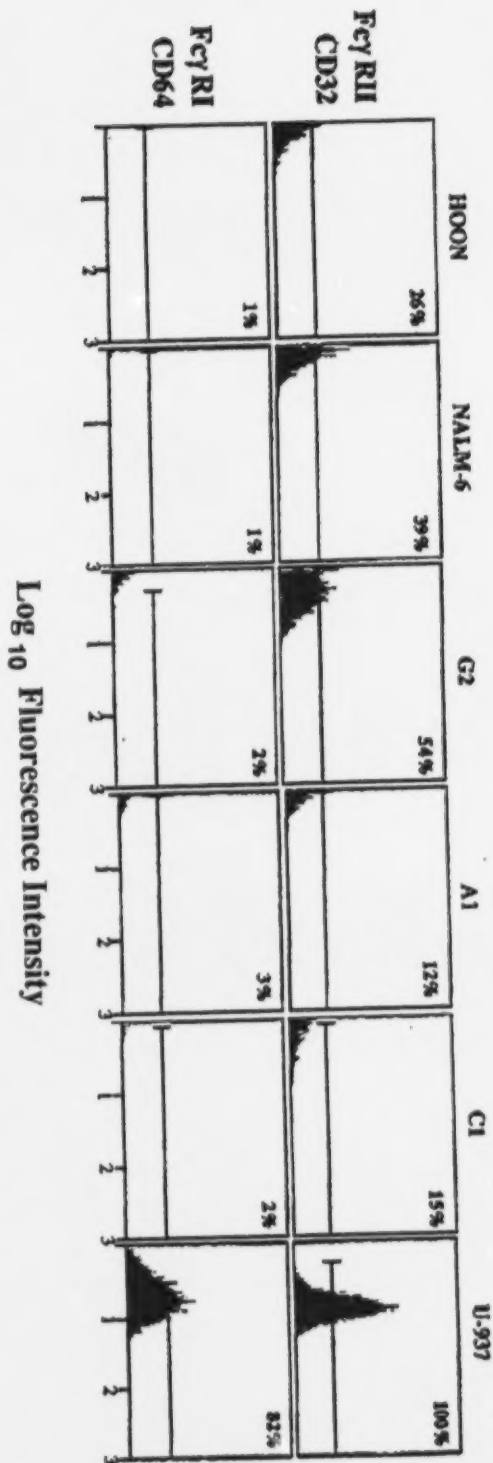


Figure 5: Surface expression of Fcγ RI and Fcγ RII on pre-B leukemic cells. The five pre-B cell lines and the U-937 pro-monocytic line were tested by flow cytometry with mAb IV.3 of IgG2b isotype, directed at the FcγRII and with mAb 197 of IgG2a isotype directed at the FcγRI. All histograms show the position of the isotype control and the percentage of cells staining positively under these conditions. All staining with IgG2a control and 197 mAb were performed in the presence of 5 ml of human AB serum to prevent binding of the Fc portion of these antibodies to the FcγRI.

Figure 5

Table I Summary of homotypic aggregation data on human leukemic pre-B cell lines

IgG	Target	Conc.	Level of aggregation induced with the following cell lines				
			HOON	NALM-6	G2	AI	CI
Antigen	(ng/ml)						
4B4	VLA-β1	12.5	5	4	2	0	1
44H6	VLA-α4	100-200	4	3	3	0	1
B-5G10	VLA-α4	(asc. 1:5000)	0	0	0	0	nd
44H6/B-5G10		12.5/12.5	0-1	0	0	0	nd
4B4/B-5G10		12.5/12.5	3	1	1	0	nd
P1D6	VLA-α5	(asc. 1:2000)	3	2	0	0	0-1
21w4	HLA-DR	100-2000	0-1	0	0	0	nd
IgG1 or IgG2b		50-500	0-1	0	0	0	0
44G4	endoglin	125-2000	0-1	0	0	0	nd
ALB6	CD9	125	4	3	3-4	0	0
B4	CD19	250	3	0	0-2	0	0
50B4	CD44	100-2000	0-1	0	0	0	nd
44D10	CD44	200-1000	0-1	0	0	0	nd
B4/ALB6		250/125	4	3	3	0	0
B4/4B4		250/125	5	4	3	0	1
B4/44H6		250/125	4	3	3	0	1
44H6/ALB6		125/125	4	3	3	0	1

All scores recorded were for cells incubated in 1% fetal bovine serum, RPMI1640, 20mM HEPES and 0.8% BSA for 20 - 24 h.

Score
0: no cells in clusters; 1: <10% in aggregates; 2: <50% in aggregates; 3: >90% in loose small clusters; 4: >90% in large clusters; and 5:

nearly 100% in large compact aggregates (43). The range of concentrations tested or the optimal concentration of mAb IgG is indicated.

Table II *Fc region involvement in mAb-induction of homotypic aggregation of pre-B cell lines*

mAb	Target	Level of aggregation induced in the cell lines	
		HOON	NALM-6
44H6 (160 ng/ml)	VLA- α 4	3, 4	3
44H6 + IV.3 (2.7 μ g/ml)		3, 4	3
ALB6 (200 ng/ml)	CD9	3, 4	3
ALB6 + IV.3 (2.7 μ g/ml)		3, 4	3
F(ab') ₂ 50H.19 (5 μ g/ml)	CD9	ND	3
4B4 (200 ng/ml)	VLA- β 1	ND	3
4B4 + IV.3 (2 μ g/ml)		ND	3
4B4 + 197 (2 μ g/ml)		ND	3
4B4 + 197 (4 μ g/ml)		ND	3

All scores were for cells incubated in 1% fetal bovine serum, RPMI 1640, 20 mM HEPES and 0-8% BSA for 20-24 hours. Score 0: no cells aggregated, 1 < 10% aggregated, 2: <50% aggregated, 3 > 90% in small clusters, 4 > 90% in large clusters (43). mAb IV.3 (anti-Fc γ RII) did not aggregate cells at 2, or 4 μ g/ml. mAb 197 (anti-Fc γ RI) did not aggregate cells at 2, or 4 μ g/ml. ND: -not done.

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IV: ANTI-CD9 MABS ENHANCE PRE-B CELL ADHERENCE TO BM-FB THROUGH A MECHANISM INVOLVING UPREGULATION OF $\alpha_4\beta_1$ AND $\alpha_5\beta_1$

A: INTRODUCTION

In humans, differentiation of B cells is confined to the bone marrow. Hematopoietic precursors display a nonrandom distribution within the bone marrow; the more immature cells concentrate at the endosteal surface while more mature cells migrate towards the main sinus (1). This may reflect interactions with particular elements within the bone marrow which are radially distributed, and which critically determine the stage of differentiation. It is becoming evident that interactions with cellular as well as extracellular components of the bone marrow stroma are required for successful lymphopoiesis. Bone marrow stromal cells produce growth factors and interleukins (2), while proteoglycans on the stromal cell surface can bind and present these growth factors to the precursor cells (3). Cellular adhesion between the precursor and cellular components of the bone marrow stroma would not only facilitate such interactions, but could generate adhesion-dependent signals which in other cells play an important role in modulating the growth response. Molecules which mediate such adhesive events have been identified and include members of the immunoglobulin gene family, integrins, and CD44 (4-6). However, the mechanisms by which adhesion is regulated and by which specificity is inferred are poorly understood.

Molecules that mediate precursor-stromal cell interactions within the bone marrow are currently being investigated. Several observations by different laboratories have implicated the integrin family of adhesive receptors as mediators of interactions between precursor cells and cellular, and extracellular components of bone marrow. For example, integrin:fibronectin interactions are important to neural crest migration (7), and to cortical thymocyte differentiation (8). Binding to fibronectin is involved in B cell maturation within

the bone marrow. Early observations implicate both RGD-dependent and RGD-independent fibronectin receptors in pre-B cell binding to fibronectin, and suggest that the differential expression of these receptors may dictate the migratory pathway of the B cell during its maturation in the bone marrow (9). A series of reports implicates the $\alpha 4 \beta 1$ integrin family member VLA-4 in both cellular and extracellular matrix interactions required for B cell maturation within the bone marrow. VLA-4 binding to its ligand VCAM plays a role in the adhesion of human pre-B cell to bone marrow fibroblasts (10). $\alpha 4$ and $\beta 1$ integrin subunits also mediate adhesion to fibronectin critical for growth and differentiation, as indicated by the inhibition of CFU-S colony formation in both spleen and bone marrow by pretreatment with an anti- $\beta 1$ mAb, or with fibronectin peptides (11), and by the inhibition of lymphopoiesis, as well as delay of myelopoiesis, in long term bone marrow cultures by mAbs against VLA-4 (12). MAb against the lymphocyte homing receptor CD44 are also potent inhibitors of lymphopoiesis in long term culture (13). Cellular adhesion within the bone marrow microenvironment therefore involves a variety of adhesion receptors with the capacity to affect cellular differentiation.

The means by which precursor cells regulate the specificity, or avidity, of their adhesion receptors during the various stages of differentiation is unclear. It has been suggested that the state of cell activation (14), as well as the presence of certain lipids (15), and cations (16) may affect integrin ligand specificity. We have recently presented evidence for a convergence of CD9, and $\beta 1$ integrin signaling pathways on pre-B cells (chapter III). CD9 is expressed on early B cells but its expression is lost with maturation, and entry into circulation (18). CD9 is once again expressed on late stage B cells (19), suggesting that the function of CD9 may be confined to a cellular microenvironment. mAbs against CD9 have the striking property of promoting both platelet and pre-B cell aggregation (20, chapter II), indicating that CD9 may regulate the function of adhesion molecules. The gene encoding CD9 has recently been cloned and found to belong to the newly described "four-pass"

superfamily of transmembrane proteins which traverse the membrane four times, in resemblance to certain ion channels (21). In this report, we present evidence that mAb against CD9 mediates adhesion between pre-B cells and bone marrow stromal elements. Furthermore, we indicate that CD9 mediated adhesion selectively involves VLA-4 and VLA-5 recognition of fibronectin, but not VLA-4 recognition of VCAM-1.

Materials and Methods:

mAb and peptides: Anti-CD9 mAb 50H.19 (22), and ALB6 (AMAC, Westbrook, Maine) are murine mAbs of the IgG2a, and IgG1 isotypes. mAbs directed against integrins include: mAb HP2/1 (AMAC, Westbrook, Maine) reacts with α_4 . mAbs A11B2 (kindly provided by Dr. C.H. Damsky, Department of Anatomy, University of California) and 4B4 (Coulter Immunology Hialeah, Florida) directed against β_1 , mAbs BIIG2, (kindly provided by Dr. C.H. Damsky) and P1D6 (Telios Pharmaceuticals) recognize α_5 . Mab 50B4, which recognizes CD44, and 44H6 which recognize VLA-4 were kindly provided by Dr. M. Letarte (Department of Immunology, University of Toronto). Anti-cellular fibronectin mAb was obtained from UBI (Lake Placid, NY). Anti-VCAM-1 mAb E1/6 was kindly provided by Dr. M. Bevilacqua (Cellular and Molecular Medicine, University of California). Peptides GRGDSP and a control peptide were obtained from Sigma (St. Louis, MO). A CS-1 and a CS-1 scrambled control peptide were the kind gift of Dr. P. Mould (Department of Biochemistry and Molecular Biology, University of Manchester Medical School).

Cells: Bone marrow aspirates (posterior iliac crest) were obtained from adult volunteers after informed consent (n=16). Sample collection was approved by the University of Alberta Hospital Ethics committee. Buffy coats were obtained by centrifugation of bone marrow aspirates at 1500 RPM for 10 minutes. Primary bone marrow fibroblasts (BM-

FB) were prepared by plating 0.5×10^6 cells/ml in alpha modified eagles medium (α -MEM) supplemented with 10% FCS, streptomycin and L-glutamine and incubated in 25cm² flasks (Falcon) at 37°C with 5% CO₂. Cultures were given one-half fluid change at day 4, after which culture medium was replaced every 7 days. The BM-FB monolayer was checked for confluency and homogeneity by inverted light microscopy. Confluent BM-FB were removed from 25cm² flasks by treatment with trypsin, seeded in 24 well plates (Falcon) and allowed to reach confluency prior to adhesion assays. The medium from each well was carefully aspirated and the monolayers washed gently with warm PBS prior to adhesion assays.

Primary bone marrow stromal cultures were prepared by plating 2.0×10^6 cells/ml in α -MEM supplemented with 12.5% FCS and 12.5% horse serum, 10^{-2} M mercaptoethanol, 10^{-6} M hydrocortisone, 1% antibiotics, 400mg/L L-glutamine and incubated in 25 cm² flasks at 37°C with 5% CO₂. The bone marrow stromal cultures were given a complete fluid change after 7 days, and thereafter given one-half fluid change every 7 days. Confluent cultures were seeded into 24 well plates as indicated for BM-FB and treated as were BM-FB cultures prior to adhesion assays.

Cell lines: The pre-B ALL cell line NALM-6 cells was obtained from Dr. B.M. Longenecker (Department of Immunology, University of Alberta) and maintained in RPMI 1640 supplemented with 5% FCS, streptomycin and L-glutamine. The pre-B ALL cell line HOON was kindly provided by Dr. M. Letarte (Department of Immunology, University of Toronto) and maintained in RPMI 1640 supplemented with 10% FCS, streptomycin and L-glutamine.

Preparation of extracellular matrices: Bone marrow stromal cultures and BM-FB cultures were prepared as described above. Confluent cultures were washed three times in PBS and incubated with 0.5% triton X-100 for 10 minutes on ice. The incubation was followed by

extensive washes with PBS to remove excess detergent prior to addition of NALM-6 or HOON. Plasma (Sigma) and cellular (UBI) fibronectin were dissolved in PBS and plated at a final concentration of 25ug/ml. Fibronectin was allowed to adhere to the wells at 4°C overnight prior to use in adhesion assays.

Adhesion assay: 1.0×10^5 NALM-6 or HOON cells in 200ul α -MEM were added to microtiter wells containing confluent BM-FB or BM-stroma. mAbs or peptides used to block adhesion were added at the initiation of culture and allowed to bind for 20 minutes at 22°C. FCS was then added to a final concentration of 5%. In pre-binding experiments, mAbs were incubated with either NALM-6 or BM-FB as indicated above. Unbound mAb was removed by three washes in PBS, and the cells were then resuspended in α -MEM to be used in the adhesion assay. FCS was titrated into the adhesion assay from 1% to 20% without significant differences in the percentage of cells binding. In adherence assays using extracellular matrix derived from bone marrow cultures, cells were resuspended in 1% BSA in α -MEM. Anti-CD9 mAbs were added to the cultures at concentrations indicated in the text and NALM-6 or HOON cells were allowed to adhere for 90 minutes at 37°C. The plates were then gently rotated on a clinical plate rotator for 5 minutes, after which the nonadherent cells were removed. The monolayers were then gently washed with α -MEM by allowing the medium to run down the sides of the wells and the plates rotated for another 5 minutes. Nonadherent cells were counted on a hemocytometer and viability assessed by trypan blue exclusion. Percent adherent cells was calculated as follows: $1 - (\text{number of free cells with mAbs} / \text{number of free cells without mAb}) \times 100$. In some experiments, NALM-6 cells were labelled with the fluorescent dye BCECF for 30 minutes at 37°C, and washed twice in PBS prior to use in the adhesion assay as indicated above. The nonadherent cells were removed as indicated above, and adherent cells were determined by exciting at a wavelength of 490 nm and reading emission at 530 nm in a Perkin Elmer spectrofluorometer 500 equipped with a microtiter plate reader.

Immunocytochemistry: BM-FB were cultured as indicated above in eight well slide chambers (Nunc, Inc. Naperville, IL). Cultures of confluent fibroblasts were washed twice in PBS, allowed to air dry overnight. Cells were incubated with primary antibody for 60 minutes at room temperature, followed by two washes in PBS. Horse radish peroxidase conjugated to goat anti-mouse mAb was added for a further 15 minutes incubation at room temperature. Freshly prepared DAB (3-3'diaminobenzidine-tetrachloride) (Sigma) in PBS containing 0.008% hydrogen peroxide was added to cells for 4-5 minutes, and the degree of staining was determined by comparison to isotype matched controls.

Immunofluorescence staining: Analysis of surface marker expression and integrins on the NALM-6 cell line was determined previously (chapter II). NALM-6 integrin expression was determined previously (chapter III), and includes $\alpha_4\beta_1$, $\alpha_5\beta_1$, and $\alpha_6\beta_1$.

In some experiments, NALM-6, in RPMI supplemented with 5% FCS, were treated with 10 ug/ml 50H.19 F(ab')₂ fragments in multi-well plates at 37°C with 5% CO₂ for 90 minutes. This concentration of 50H.19 F(ab')₂ induced homotypic aggregation of both NALM-6 and HOON. Integrin expression was determined by addition of anti-integrin mAbs prior to addition of anti-CD9 mAb, or post adhesion assay. FITC conjugated goat anti-mouse antibody (Fc specific) was added at 0.1 mg/ml for 30 minute on ice. Cells were washed three times in PBS. Samples were analyzed on a FACSCAN (Becton Dickinson) using Lysis II program. All experiments included staining with isotype-matched control antibodies. Ten thousand cells were analyzed from each sample.

Results:

1. NALM-6 binding to bone marrow adherent cell cultures

Pre B cell differentiation requires close interaction with bone marrow stroma (23). We investigated the ability of human bone marrow stromal cultures to support the adhesion of NALM-6 and HOON cells. As shown in Fig. 1, NALM-6 and HOON cells bound well to bone marrow stromal cultures (BM stroma). We next determined whether specific bone marrow components supported NALM-6 adhesion. Bone marrow-derived fibroblasts (BM-FB) were able to support NALM-6 and HOON adhesion better than were bone marrow stroma. Purified cellular fibronectin, at 25ug/ml, or ECM deposited by BM stroma and BM-FB, were unable to support the adhesion of either NALM-6 or HOON.

2. Anti-CD9 mAbs enhance pre-B cell binding to BM-fibroblasts:

We have previously shown that mAb modulation of CD9 results in homotypic aggregation of pre-B cell lines NALM-6 and HOON (chapter I). We wished to determine whether CD9 modulation by mAb induced heterotypic interactions with components present within the bone marrow microenvironment. NALM-6 cells were cultured in the presence of anti-CD9 mAbs 50H.19 and ALB6, and their adhesion to BM-AD, and BM-FB cultures was determined. Addition of less than 1ug/ml of 50H.19 and 0.2 ug/ml ALB6 mAbs enhanced NALM-6 adhesion to BM-fibroblasts greater than 200% (Figure 2). The same concentration of 50H.19 and ALB6 did not enhance NALM-6 to BM-AD, or to ECM derived from BM-AD or BM-FB. Addition of 2.5ug/ml F(ab')₂ fragment of 50H.19 enhanced NALM-6 binding to BM-FB by 180%, (56% adhesion) suggesting that Fc receptors are not involved. The enhanced adhesion was not blocked by 4ug/ml IV.3 directed against the FcγII receptor, further suggesting the lack of Fc receptor involvement. Furthermore, CD9 enhanced adhesion was not blocked by the protein kinase C (PKC) inhibitors H7 or staurosporine (68% and 70% adhesion). This finding suggest that CD9

enhanced adhesion, unlike adhesion mediated through LFA-1, may proceed through a pathway which does not involve the activation of PKC, and as such, may be indicative of a novel mechanism for integrin regulation.

3. Analysis of adhesion receptor expression on NALM-6 and bone marrow-derived fibroblasts:

Interactions of cells with other cells, or with components of the extracellular matrix is mediated by multiple adhesion receptors which include i) integrins, ii) CD44 and iii) members of the immunoglobulin supergene family (eg. VCAM-1). The expression of VLA integrins, and CD44 on NALM-6 and HOON were previously shown as indicated in (chapter II). NALM-6 weakly expresses β_2 , and is negative for ICAM-1 (chapter I). We show here that NALM-6 does not express VCAM-1 or β_7 (figure 3a).

Figure 3 also shows that antibody modulation of CD9 does not alter integrin expression. Since anti-CD9 mAb enhanced NALM-6 adhesion to BM-FB, we investigated whether antibody modulation of CD9 alters the level of integrin expression. F(ab')₂ fragments of mAb 50H.19 were employed to induce cells so that integrin expression could be quantitated by a secondary mAb directed against the Fc region of mouse immunoglobulin. As shown in figure 3b, induction of aggregation by mAb 50H.19 F(ab')₂ does not result in either the upregulation, or downregulation, of β_1 , α_4 or α_5 integrin subunit expression.

BM-FB expressed the collagen/laminin receptor α_2 and the fibronectin receptor α_5 , but not α_3 , α_4 or α_6 . The BM-FB did not express detectable levels of β_2 , β_3 , or β_7 , but strongly expressed β_1 and VCAM-1. BM-FB were negative for CD9, CD44 and the endothelial cell marker Factor VIII, however, they did contain surface bound cellular fibronectin (Table I).

4. Anti-CD9 mAb enhanced adhesion can be blocked by antibodies against β_1 , $\alpha_4\beta_1$ and $\alpha_5\beta_1$:

To investigate whether CD9-induced enhancement was mediated through upregulation of adhesion receptors, we incubated NALM-6 with a panel of blocking mAbs. As indicated in Figure 4a, the anti- β_1 mAb, AIIB2, completely inhibited anti-CD9 enhanced adhesion, as well as basal level adhesion. An anti- $\alpha_4\beta_1$ mAb, HP2/1, blocked adhesion by 60%, whereas the anti- $\alpha_5\beta_1$ mAb BIIG2 inhibited adhesion by 32%. However, mAb BIIG2 co-incubated with HP2/1, blocked anti-CD9 enhanced adhesion by 100%, indicating that anti-CD9 enhanced adhesion involves both the α_4 and α_5 integrins. Basal level adhesion was not blocked by the anti- $\alpha_5\beta_1$ mAb BIIG2, however, the anti- $\alpha_4\beta_1$ mAb HP2/1 blocked binding by 40%. An anti-VCAM-1 mAb E1/6 also inhibited anti-CD9 enhanced adhesion by 14%. Since basal level adhesion was also inhibited by E1/6 (24%) it implies that the recognition of VCAM-1, by $\alpha_4\beta_1$, is not upregulated by the anti-CD9 mAbs (figure 4a).

To determine which integrins on the NALM-6 and BM-FB were participating in adhesion, we pre-incubated either NALM-6 or BM-FB's with blocking antibodies and observed anti-CD9 mAb enhanced adhesion. When BM-FB's were pre-incubated with anti- β_1 mAbs 4B4 or AIIB2, CD9 enhanced adhesion was no longer blocked. However, if NALM-6 cells were pre-incubated with mAbs 4B4 or AIIB2, greater than 80% inhibition was observed, suggesting that β_1 integrin on NALM-6 was mediating the enhanced adhesion. Similarly, pre-incubation of NALM-6 with mAbs HP2/1 and BIIG2, recognizing α_4 and α_5 , blocked adhesion. Preincubation with mAb HP2/1 resulted in greater than 50% inhibition and mAb BIIG2 resulted in greater than 30% inhibition. Pre-incubation of BM-FBs with BIIG2 reduced adhesion 7%, whereas pre-incubation with HP21 was ineffective (figure 4b). These results suggest that CD9 is regulating adhesion receptors on NALM-6,

as opposed to regulating an indirect mechanism, such as release of factors, to alter the avidity of $\alpha 4\beta 1$ and $\alpha 5\beta 1$ on BM-FB.

5. Synthetic peptides of fibronectin block anti-CD9 enhanced adhesion:

$\alpha 4\beta 1$ and $\alpha 5\beta 1$ bind to fibronectin at distinct and nonoverlapping sites (24, 25). To determine which domain of fibronectin was involved in the anti-CD9 enhanced adhesion we utilized a synthetic peptide containing the cell attachment sequence RGD and a peptide derived from the type III connecting segment (III-CS) containing the CS-1 domain (24). RGD has been demonstrated to be the attachment site for $\alpha 5\beta 1$, while CS-1 is a binding site for $\alpha 4\beta 1$. As indicated in Table III, the CS-1 peptide blocked anti-CD9 enhanced adhesion by 49%, even at concentrations which allow maximal for inhibition of adhesion to a CS-1 substrates (25). However, a scrambled CS-1 peptide (CS-1s) was ineffective. The synthetic peptide, Gly-Arg-Gly-Asp-Thr-Pro, inhibited anti-CD9 enhanced adhesion by 30% at a concentration of 30ug/ml, while control peptides were ineffective. Both the CS-1 and RGD containing peptides did not inhibit basal level binding. A mAb against cellular fibronectin, which efficiently stained BM-FB, was unable to block anti-CD9 enhanced adhesion. $\times$ However, this data does suggest that anti-CD9 mAb enhanced adhesion is mediated through upregulation of $\alpha 4$ and $\alpha 5$ recognition of fibronectin.

Discussion:

The study of B lymphopoiesis has been greatly enhanced by the advent of long term bone marrow cultures. Murine B cell lymphopoiesis can be observed in Whitlock-Witte cultures (26). While conditions permitting human B lymphopoiesis *in-vitro* have yet to be found, the use of *in-vitro* B cell differentiation systems greatly assist in the delineation of cellular interactions which occur between stromal cell and progenitor cell. In this chapter, evidence

is presented that mAb modulation of CD9 enhance pre-B cell binding to bone marrow fibroblasts. We provide evidence suggesting that CD9 mediates adhesion of pre-B cells to fibronectin through the selective regulation of $\alpha 4\beta 1$ and $\alpha 5\beta 1$ recognition of fibronectin.

We have implicated VLA integrins in anti-CD9 mAb enhanced BM-FB interactions, as this adhesion is blocked by mAbs against the common integrin $\beta 1$ chain. The mechanism of CD9 mAb enhanced adhesion involves $\alpha 4\beta 1$ and $\alpha 5\beta 1$ recognition of fibronectin. The involvement of both $\alpha 4\beta 1$ and $\alpha 5\beta 1$ in fibronectin recognition is suggested by observations that blocking mAbs in combination completely inhibit anti-CD9 enhanced adhesion, whereas both mAbs used individually result in only partial inhibition. Since anti-CD9 mAb enhanced adhesion was blocked by a peptide containing the CS-1 domain of FN, and with a synthetic peptide containing the sequence RGD, whereas basal level binding was not blocked by either of these peptides, it indicates that addition of anti-CD9 mAbs results in adhesion which is qualitatively, as well as quantitatively different from basal level binding. Although concentrations of RGD peptides greater than 300ug/ml have been reported to inhibit $\alpha 4\beta 1$ binding to the CS-1 domain (27), the concentrations used in this study were below those shown to compete for the CS-1 site. It is therefore more likely that the RGD peptide is exerting its effect on ALL adhesion to the BM-FB by binding to the CCBD (central cell binding domain) domain of FN.

It has been suggested that since fibronectin contains multiple adhesion sites which may generate functional signals, integrin recognition of a particular domain may be dictated by receptor conformation (28). Our evidence indicates that CD9 regulates the functional interaction of $\alpha 4\beta 1$ and $\alpha 5\beta 1$ integrins with fibronectin, in the absence of an increased density of $\beta 1$ or α chain ($\alpha 4$ and $\alpha 5$) expression. It is of considerable interest that the anti-CD9 mAbs do not detectably alter $\alpha 4\beta 1$ recognition of VCAM-1, while enhancing $\alpha 4\beta 1$ recognition of fibronectin. This finding suggests that CD9 serves to regulate the specificity

of integrin:ligand interactions. The mechanism by which CD9 modulates integrin specificity is unknown, but presumably involves alterations in integrin conformation to expose binding sites for neo-ligands. Although the mechanisms governing integrin stereoselectivity are not well understood, several studies have described enhancement or alteration of integrin:ligand interactions induced by mAb, lipids, cations or cell activation (29-36), suggesting that integrins are subject to multiple modes of regulation. The mechanism by which CD9 stimulation regulates integrin function is unclear, but could involve direct interaction between CD9 and the integrin. A precedence for this idea is derived from evidence for an association between CD9 and β_3 integrins (37). More recently, we have shown that the ability of β_1 mAb induced pre-B cell aggregation correlates with the presence of CD9, suggesting that CD9 and β_1 signalling pathways may converge (chapter III). Since CD9 is a potent promotor of platelet aggregation in the absence of Fc receptor occupation (38), CD9 appears to function as a signal transducing antigen. Taken together, these data suggest that CD9 may regulate the conformation of neighboring integrins with which it associates.

There is considerable evidence to support the concept that the state of cell activation regulates the avidity of integrins for their respective ligand through the generation of intracellular signals. However, the mechanism of VLA upregulation is unclear. T cell adhesion to purified ECM protein, ICAM, or VCAM-1 is rapidly upregulated after activation, in the absence of increased integrin cell surface expression (33, 34). A brief stimulation of NK cells by PMA results in an increased binding to laminin, and is accompanied by the phosphorylation of α_6 , suggesting that PKC activation and subsequent phosphorylation events may be involved in $\alpha_6\beta_1$ upregulation (39). The VLA-4-dependent binding of B cells and eosinophils to fibronectin and VCAM-1, is upregulated upon activation despite the constitutive expression of VLA-4 on both cell types, indicating a rapid qualitative change in the avidity of VLA-4 for its ligand (40, 41). Our earlier findings that

activation with PMA failed to induce homotypic aggregation of pre-B cell lines (chapter II), coupled with the inability of PKC inhibitors to block CD9 enhanced adhesion to BM-FB, indicates that pre-B cells may regulate integrin-avidity by utilizing alternative mechanisms to those which function in mature circulating T cells and B cells. Interestingly, CD4 and the CD4-associated tyrosine protein kinase *lck* has been implicated the transduction of signals leading to the activation of fibronectin and laminin receptors on murine T lymphocytes (42). While many cell types lack CD4, other molecules may be involved which serve to transmit signals leading to the upregulation of integrin activities. The results presented in this report suggest that CD9 may selectively regulate integrin-mediated binding of pre-B cells to fibronectin on the surface of the bone marrow fibroblasts.

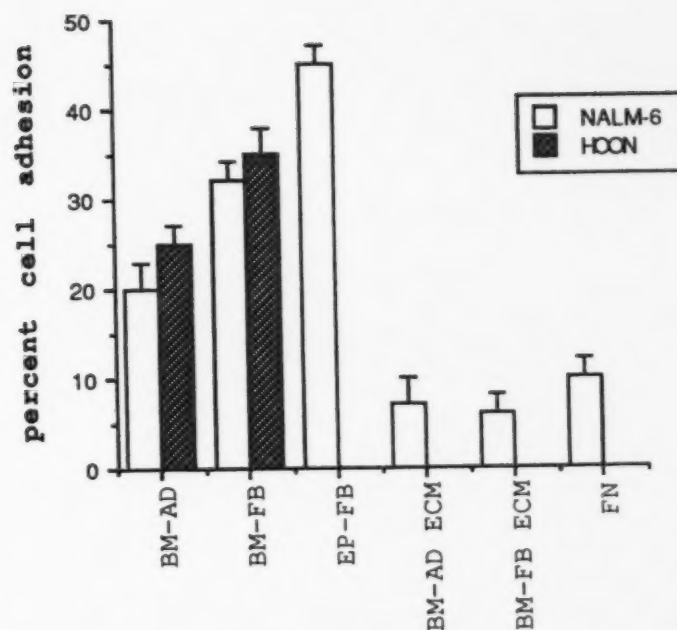
Fibronectin is a multifunctional glycoprotein which supports a wide variety of cellular activities such as cell adhesion, migration, proliferation and differentiation. We used anti-cellular fibronectin mAbs as well matrices composed of purified fibronectin to demonstrate pre-B cell adhesion to fibronectin. The anti-fibronectin mAbs directed against the cell binding site as well as the COOH-terminal of fibronectin blocked adhesion, implicates two distinct cell binding sites of fibronectin in CD9 enhanced adhesion. The inability of purified cellular fibronectin to support NALM-6 adhesion is intriguing and might indicate the presence of other components within BM-FB cultures, such as ECM proteins, cell surface proteoglycans, gangliosides, and receptors, which may be actively involved in the adhesion process. The interaction of CD44 with hyaluronan is critical to lymphopoiesis in long term bone marrow cultures (43), however, we did not observe any inhibition by mAb 50B4 (directed against CD44). Furthermore, we were unable to inhibit CD9 enhanced adhesion with an anti- β_2 mAb, suggesting that LFA-1-ICAM interactions are not involved. Although we cannot define other components which may be involved in CD9 enhanced adhesion, our results suggest that VLA-4 and VLA-5 are the major mediators of CD9 enhanced pre-B cell binding to cell surface bound fibronectin. The involvement of multiple

fibronectin adhesion receptors may reflect a system which requires a complexity of adhesion receptors to confer cell binding specificity. Extracellular components serve not only as an anchor for cell adhesion, but also transmit information regarding the immediate cellular micro-environment. Thus, the selective binding of VLA-4 to the CS-1 site within fibronectin, and not to VCAM-1, and the combined upregulation of VLA-4 and VLA-5, may provide information which instructs the cell towards a particular functional response. CD9 may not only increase the affinity of the receptor but also allow it to form a multivalent complex able to bind fibronectin more avidly.

The data we have presented is based primarily on experiments using two non-T ALL cell lines. In this report, we corroborate the finding by Ryan et al. (10) that BM-FB support NALM-6 adhesion (defined here as basal level adhesion). Furthermore, Ryan et al. show that normal bone marrow cells, and NALM-6 basal level adhesion are both mediated by VLA-4. Evidence from Freedman et al. indicate that NALM-6 cells adhesion to germinal centers is mediated via VLA-4 and Incam-110 (VCAM-1), a mechanism identical to that utilized by activated normal B cells binding to endothelial cells (44). Neutrophil binding to endothelial cells can be enhanced by mAbs against CD9, consistent with results presented here (45). These data would suggest that the cell line NALM-6 exhibits similar adhesive properties compared to normal cells. In a recent study, CD10⁺ ALL cells have been shown to exhibit a similar level of binding to BM-FB compared to normal CD10⁺TdT⁺ B cell precursors (46). Furthermore, both ALL cells and normal B cell precursors bind to BM-FB through a VLA-4/VCAM-1 interaction. These findings suggest that the adhesive process regulated by β_1 integrins are not altered in the leukemic cell, and that perhaps alterations in the regulation, or function of other receptors may be a determining factor in the progression of the leukemic phenotype.

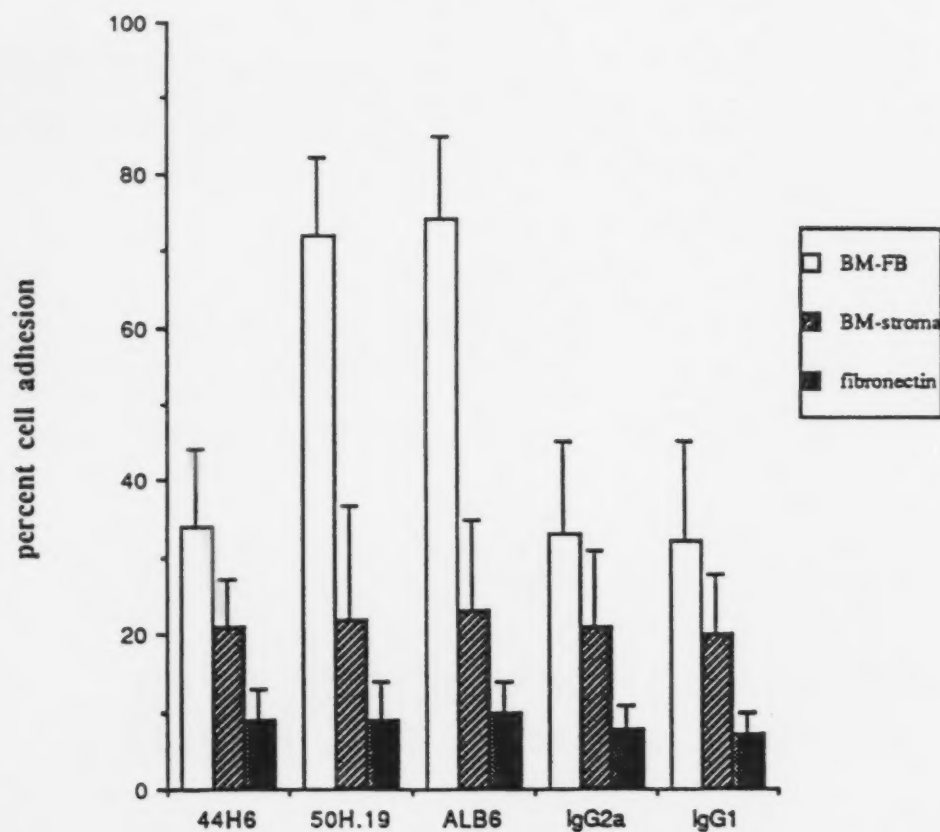
Maturation of B cells appears to involve spatial and temporal interactions with cellular and extracellular components within the bone marrow. We have shown that modulation of CD9 by mAbs renders functional two pre-B fibronectin receptors ($\alpha 4\beta 1$ and $\alpha 5\beta 1$), to specifically regulate adhesion of pre-B cells to fibronectin on the surface of bone marrow fibroblasts. Recognition of the various components involved in progenitor cell:stromal cell communication will further our understanding of B cell differentiation and may delineate changes which contribute to the leukemic phenotype.

Figure 1



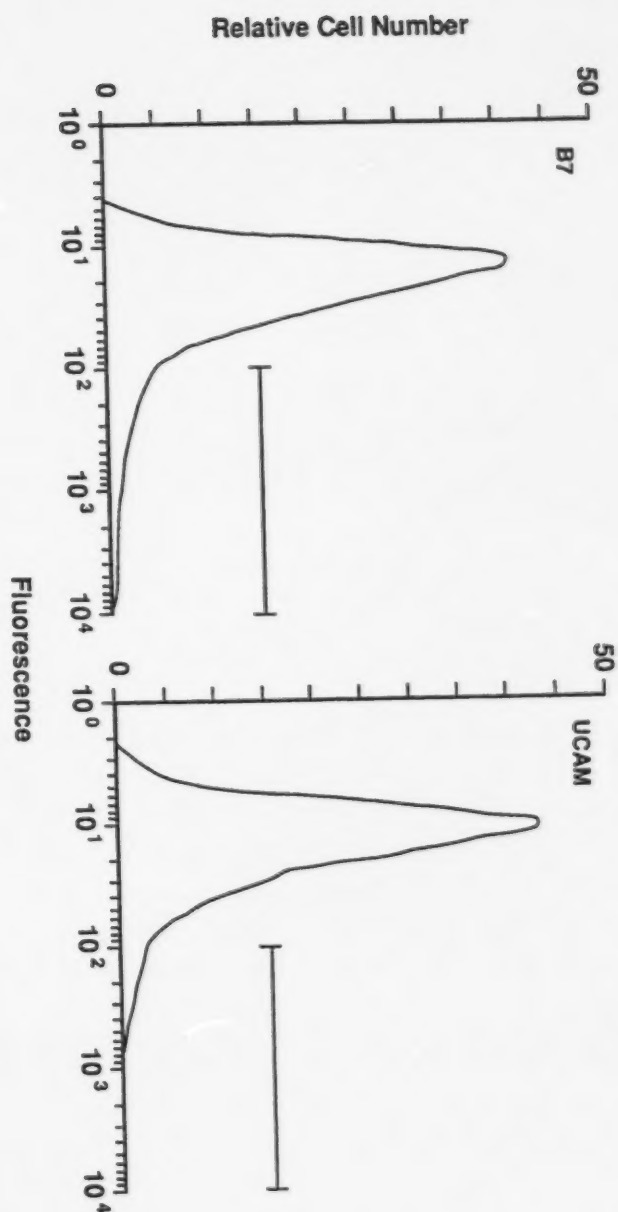
1. *Binding of NALM-6 and HOON to BM-FB* 1.0×10^5 NALM-6 or HOON cells were incubated on the following: confluent BM-FB (n=8), BM stromal cells (BM stroma n=3), extracellular matrix (ECM) deposited by BM-FB or BM stroma (n=3) or 25ug/ml fibronectin for 90 minutes at 37 C (n=4). Percent adherence was determined as indicated in materials and methods.

Figure 2



2. *Anti-CD9 mAbs enhance preB cell adherence to BM-FB:* 1.0×10^5 NALM-6 cells were plated in wells containing confluent BM-FB, (n=8), BM stromal cells (BM-stroma n=3), or 25ug/ml fibronectin (n=4) prepared as indicated in materials and methods. The following mAbs were added and the adherent assay was carried out as indicated in materials and methods: anti-CD9 mAbs 50H.19 (1ug/ml), ALB₆ (0.2ug/ml), anti-VLA-4 mAb 44H6 (1ug/ml), and mouse myeloma mAbs of IgG2a (1ug/ml) and IgG1 (1ug/ml) isotype controls. Concentrations of anti-CD9 mAbs used were found to promote homotypic aggregation of pre-B cell lines.

Figure 3a



3. A) Adhesion receptors on NALM-6 NALM-6 were stained according with anti-VCAM-1 mAb E1/6 and anti-B7 mAb Act-1, as indicated in materials and methods. Relevant Ig isotypic controls were used.

Relative Cell Number

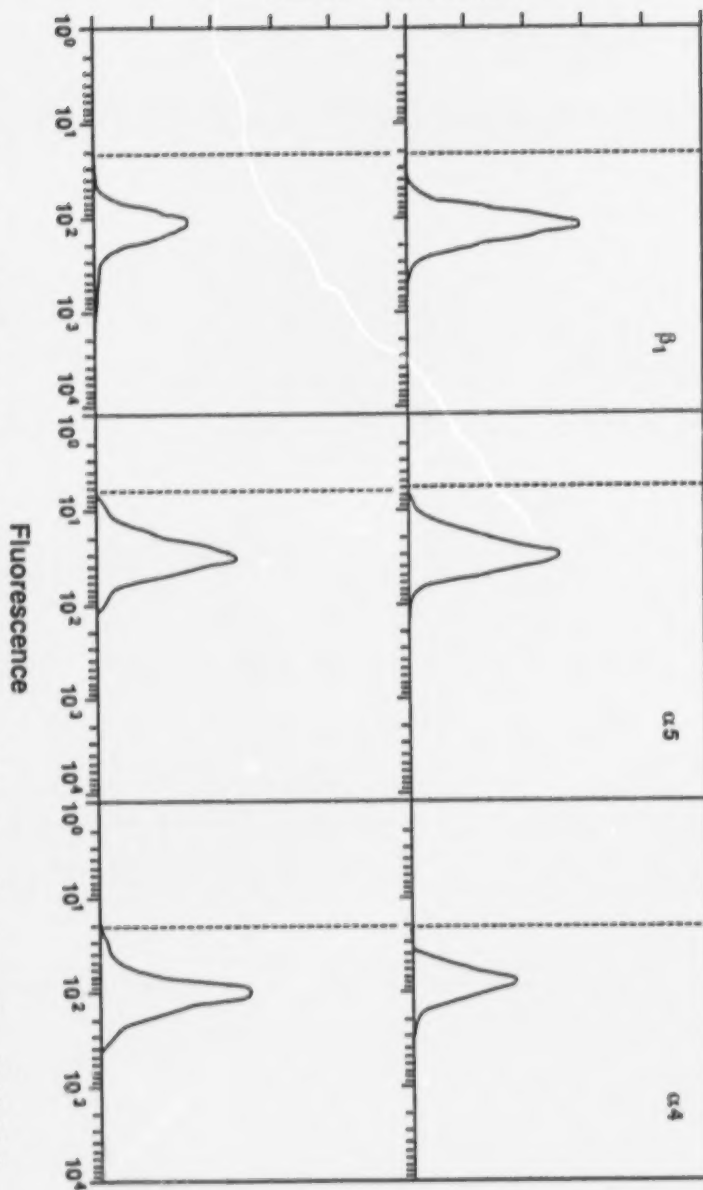
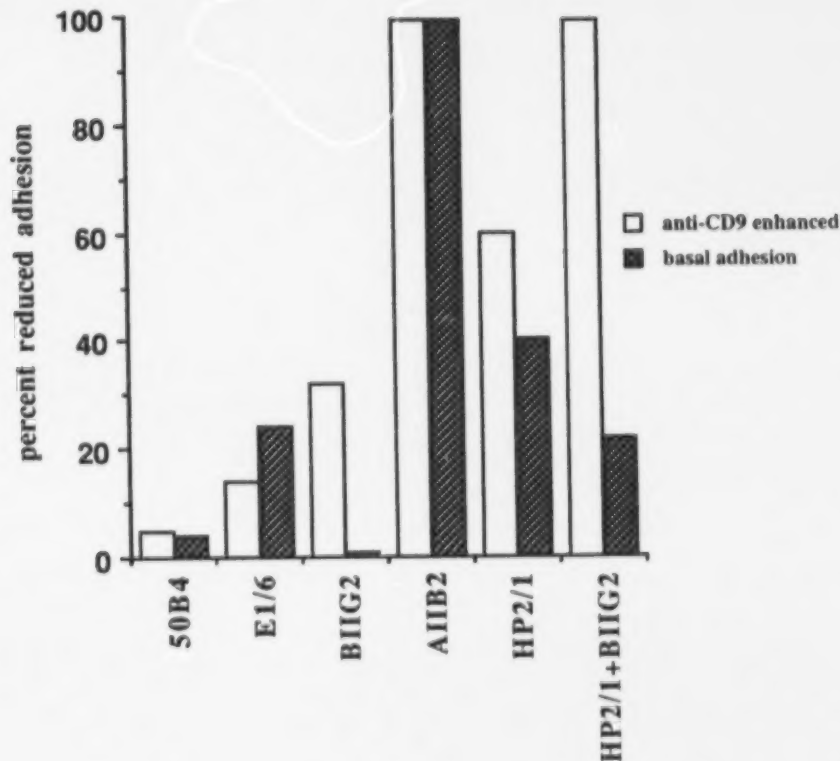


Figure 3b

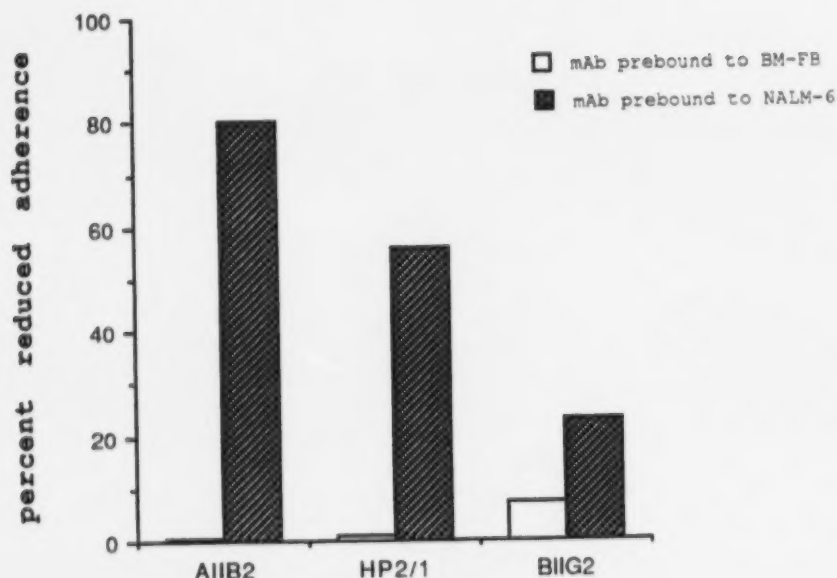
3. B) *Binding of anti-CD9 mAb F(ab)² does not alter integrin expression* NALM-6 were analyzed for integrin receptor expression before and after mAb modulation of CD9. Cells were incubated with 10ug/ml 50H.19 F(ab)², a concentration known to promote NALM-6 homotypic aggregation. 2 hours post incubation cells were analyzed for integrin expression as indicated in materials and methods. Mabs used were 4B4 (anti- β_1), 44H6 (anti-VLA-4) or PID6 (anti-VLA-5) mAbs as indicated in materials. Solid line indicates integrin expression at time 0 and dashed line indicated integrin expression after 2 hour incubation with 50H.19 F(ab)². Mean fluorescence channels before and after incubation are: 4B4 (553 and 557), 44H6 (469 and 532), and PID6 (341 and 394).

Figure 4a



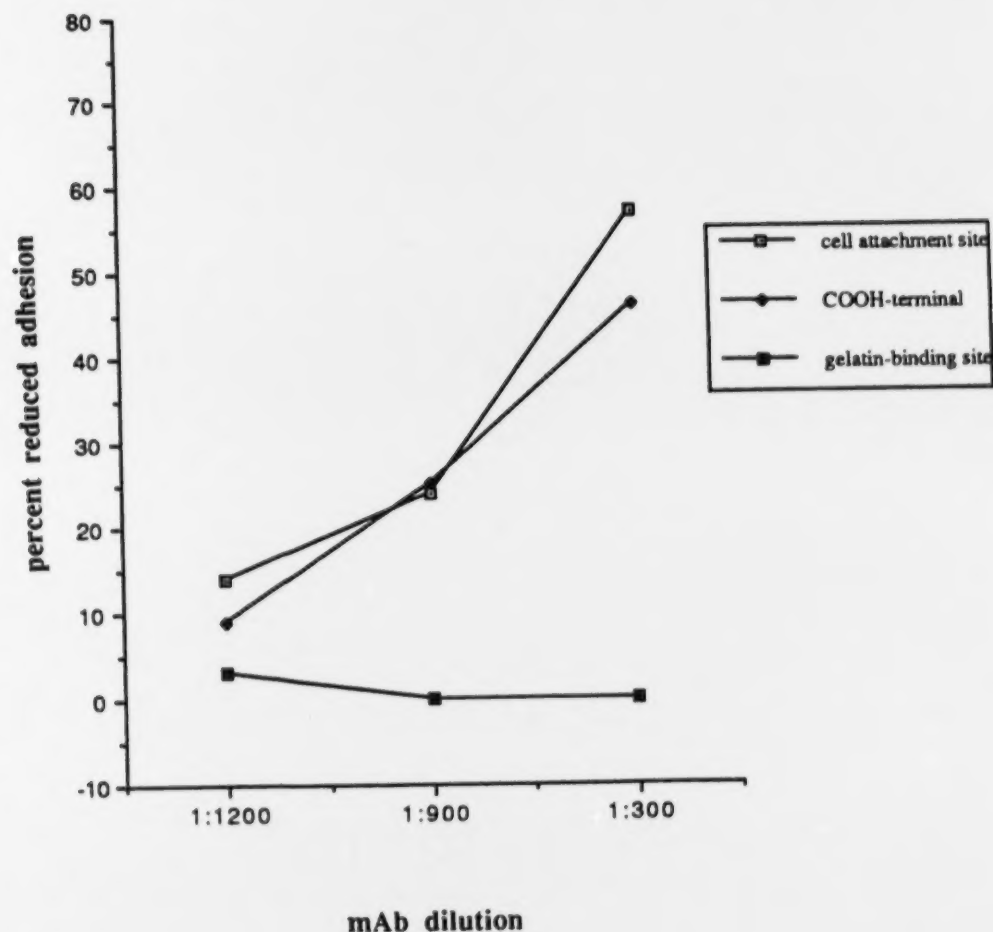
4 .A) mAbs against $\beta 1$ integrins VLA-4 and VLA-5 inhibit anti-CD9 enhanced adhesion to BM-FB 1×10^5 NALM-6 cells and confluent BM-FB cultures, in a-MEM without FCS, were treated with the following integrin mAbs: HP2/1 (1ug/ml), BIIG2 supernatant (1:5) and AIIB2 supernatant (1:5), and anti-VCAM-1 mAb E1/6 (5ug/ml) for 20 minutes at room temperature. After incubation, FCS was added to a final concentration of 5%, and anti-CD9 mAb 50H.19 was added to a final concentraion of 0.125ug/ml. An IgG1 isotype control mAb and an irrelevant culture supernatent control did not inhibit anti-CD9 mAb enhanced adhesion.

Figure 4b



4. B) *Integrins on NALM-6, but not on BM-FB mediate anti-CD9 enhanced NALM-6 adhesion* 1×10^5 NALM-6 cells, or confluent monolayers of BM-FB, in a-MEM without FCS, were incubated with HP2/1 (1ug/ml), BIIG2 supernatant (1:5) or AIIB2 (1:5), for 20 minutes at room temperature. After 20 minutes, the cells were washed three times in PBS, resuspended in a-MEM with 5% FCS and 0.125ug/ml anti-CD9 mAb 50H.19 and the adherence assay carried out as indicated in materials and methods. An irrelevant purified IgG1 and irrelevant culture supernatant were used as negative controls.

Figure 5



5.) *Anti-fibronectin mAbs inhibit NALM-6 adhesion to BM-FB:* BM-FB, grown to confluency as indicated in materials and methods, were washed twice in PBS, and incubated with the indicated dilutions of anti-fibronectin mAbs for 20 minutes at room temperature. The antibodies were diluted in PBS supplemented with 1% BSA. After incubation the medium was aspirated and 1.0×10^5 NALM-6 cells were added in a-MEM supplemented with 1% BSA. ANti-CD9 mAb 50H.19 (0.5 ug/ml) was also added in conjunction with NALM-6 cells. The adhesion assay was carried out as indicated in materials and methods. The anti-fibronectin mAbs used were: 1) a mAb directed against the cell attachment site, 2) a mAb against the COOH-terminal and 3) a mAb against the gelatin-binding site.

TABLE I: Expression of adhesion receptors on BM-FB

	$\alpha 1$	$\alpha 2$	$\alpha 3$	$\alpha 4$	$\alpha 5$	$\alpha 6$	$\beta 1$	$\beta 2$	$\beta 3$	$\beta 7$	VCAM-1	CFN	FVIII	CD9	CD44
BM-FB	++	++	-	-	+	-	++	-	-	-	++	+++	-	-	-

Bone marrow fibroblasts, grown to confluency in chamber slides, were analyzed for the above indicated adhesion receptors by indirect immunocytochemistry using the anti-adhesion receptor mAbs indicated in materials and methods, and an HRP-goat anti-mouse. Isotype matched mAbs were used as negative controls when determining positive reactivity.

Table II : Fibronectin peptides block anti-CD9 enhanced adhesion to BM-FB

	<u>% reduced adhesion to BM-FB</u>
CS-1	50
CS-1s (control)	12
RGD	30
RAD (control)	0.5
CS-1 + E1/6	76
CS-1s + E1/6	22
RGD + E1/6	50
RAD + E1/6	28

Confluent bone marrow fibroblasts were pretreated with either 500ug/ml CS-1 and CS-1s control peptide, 30ug/ml RGD (Gly-Arg-Gly-Thr-Pro) or RAD control (Gly-Arg-Ala-Asp-Ser-Pro-Lys) peptide, 10ug/ml anti-VCAM-1 mAb E1/6, in α -MEM without FCS, prior to addition of Nalm-6 cells and anti-CD9 mAb ALB6 0.4ug/ml. Adhesion of Nalm-6 to bone marrow fibroblasts was carried out as indicated in materials and methods.

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V: ANTI-CD9 MABS INDUCED DEVELOPMENT OF DENDRITIC CELLS FROM CD9+ PRECURSORS IN PERIPHERAL BLOOD REQUIRES VLA-5 AND CD44

A. INTRODUCTION

The immune system contains a network of specialized antigen presenting cells, termed dendritic cells, which can sequester and present antigen to T cells(1). Dendritic cells have been characterized in lymphoid and nonlymphoid organs and include the interdigitating cells of lymphoid organs (lymph node(2), spleen(3), thymus(4), tonsil(5), veiled cells of peripheral blood and lymph (6,7), langerhans' cells of the epidermis (8), and dendritic cells along lung airway epithelium (9). Whether dendritic cells within the various organs arise from a common precursor or whether they represent separate cell lineages is unclear. However, dendritic cells display characteristics of morphology and function that are useful in their identification. Upon isolation, dendritic cells have continuously extending lamellipodia or veils, a characteristic not common to lymphocytes or macrophages.(10). Phenotypically, dendritic cells are rich in class II MHC antigen, although they lack markers of lymphoid or myeloid lineage cells such as CD3, and CD14 (11). Consistent with the two features described above, dendritic cells are potent stimulators of MLC in vitro, as compared to monocytes or B cells (12).

DC precursors originate within bone marrow (13), but have also been isolated from peripheral blood, presumably enroute to lymphoid and nonlymphoid tissue. The identification of the dendritic cell precursor has been extensively investigated as a means to determine the relationship between DC and the various hematopoietic lineages. To this end, cells morphologically and phenotypically resembling Langerhans cells were described in methycellulose cultures of human peripheral blood and bone marrow. Langerhans cells appeared in colonies which also contained macrophages, suggesting that the dendritic cell

and the macrophage may have a common myeloid precursor (14). Corradi et al. earlier observed that peripheral blood cells bearing the B cell marker CD20 gave rise to dendritic cells upon stimulation with phorbol ester, which suggests that at least some types of dendritic cells may belong to the B cell lineage (15). Recently, Inaba et al. reported isolation of dendritic cells from precursors present in mouse peripheral blood (16). Upon the addition of GM-CSF, peripheral blood precursors formed proliferating aggregates in culture. These aggregates gave rise to non-proliferating cells which morphologically, and functionally resembled dendritic cells. Analysis of cell surface antigens expressed by both precursor cells and their progeny indicated the lack of B cell and myeloid markers. The reports cited above describe the development of dendritic cells from precursors which require exogenous stimulus either in the form of exogenous growth factors or activation by mitogen.

In this chapter, we describe the differentiation of dendritic cells derived from transiently adherent peripheral blood cells. This differentiation is dependent on modulation of the surface antigen CD9 by anti-CD9 mAbs. CD9 is a member of a novel family of structurally related molecules, including CD63, CD51, CD37, TAPA-1, R2, SM-23 and Co-029 (17), which has been shown to mediate cell-cell interactions. Although the biological function is unknown, mAbs against CD9 initiate platelet signaling pathways leading to platelet activation and aggregation (18,19), as well as aggregation of pre-B ALL cell lines (chapter II) in a β_1 integrin dependent manner (chapter III). We also present evidence that CD9 may induce signals effecting dendritic cell differentiation from a peripheral blood precursor through a process which involves multiple adhesion receptors.

B. MATERIALS AND METHODS

Monoclonal Antibodies and Peptides: Anti-CD9 mAbs used were 50H.19 (20) and ALB6 (AMAC, Westbrook, Maine). 7H.3 and I2 (anti-HLA-Dr), and 9H.1 (anti-B2 microglobulin) were gifts of Dr. M. Longenecker (Department of Immunology, University of Alberta). Other antibodies include: Leu 4 (CD3) LeuM3 (CD14), Leu3a (CD4), Leu2b (CD8) Leu6 (CD1), Hle-1 (CD45) (Becton Dickinson, CA.), and B4 (CD19), B1 (CD20), B2 (CD21), I2 (HLA-Dr), CD11a, Mo-1(CD11b), 4B4 and T1 (CD5) (Coulter Immunology, Ont.). Mab CD11a and CD18 was obtained from AMAC. MAb P1D6 ($\alpha 5\beta 1$) and a polyclonal anti-fibronectin receptor antibody were obtained from Telios Pharmaceutical. Anti- $\alpha 4\beta 1$ mAbs include P4G9 (Telios Pharamaceutical) and HP2-1 (AMAC). Mabs TS2/7($\alpha 1\beta 1$) was a gift of Dr. M. Hemler. mAbs P1E6 ($\alpha 2\beta 1$), P1B5ew ($\alpha 3\beta 1$) were gifts of Dr. E. Wayner. mAb J1B5 ($\alpha 6\beta 1$) was a gift of Dr. C. Damsky. mAbs 50B4 (CD44) 44H6 ($\alpha 4\beta 1$) were gifts of Dr. M. Letarte (Department of Immunology, University of Toronto). mAb Act-1 directed against $\beta 7$ was the kind gift of Dr. S. Shaw (EIB-NCI, NIH). Antibody recognizing the cytoplasmic S-100 marker was a gift of Dr. S. Poppema (Department of Pathology, Cross Cancer Institute). Peptides RGDdSP and RGEsp were obtained from Telios Pharmaceuticals. The CS-1 synthetic peptides (containing residues 1-25 of the IIIICS region of fibronectin)(), and a control scrambled peptide were gifts of Dr. P. Mould (Department of Biochemistry and Molecular Biology, University of Manchester Medical School).

Culture Medium: All cultures were in RPMI 1640 (Gibco Laboratories, Grand Island NY) supplemented with 10% FCS (Gibco), streptomycin, and L-glutamine. Cells plated on matrices were cultured in RPMI-1640 supplemented with 1% BSA and 1% FCS, streptomycin and L-glutamine.

Reagents: PMA and cytochalasin B were dissolved in ethanol before addition to cultures. LME was dissolved in serum-free RPMI-1640 prior to use.

Preparation of extracellular matrix: Human plasma fibronectin and BSA were obtained from Sigma and solubilized in PBS prior to plating at 25 ug/ml. Peptide 2000 containing the RGD sequence was obtained from Telios Pharmaceuticals (San Diego, CA.), and solubilized in 70% ethanol prior to plating at 25ug/ml. Microtiter wells (NUNC) wells containing FN, BSA or Peptide 2000 were incubated at room temperature for 2 hours. The wells were then washed twice with PBS and immediately used for cell culture. Collagen type IV (Sigma) was prepared in PBS and plated at 10ug/ml. Wells coated with collagen were allowed to dry overnight and rehydrated with PBS prior to plating of cells. Laminin (Sigma) was prepared in PBS and plated at 5ug/ml overnight at room temperature. In some experiments, reconstituted collagen was then incubated with 5ug/ml laminin (Sigma) for 1 hour 37°C. The wells were washed twice with PBS and used for cell culture. Hyaluronan (HA) (Sigma cat no. H-1751) was reconstituted in distilled water prior to addition to transiently adherent cell cultures.

Cell separation: Whole peripheral blood was obtained from healthy adult donors by venous puncture and collected into heparin-treated vacutainer tubes. Peripheral blood mononuclear cells (PBMC) were isolated on Ficoll-Hypaque density gradients ($d=1.077$). Cells at the interface were collected and washed once in cold phosphate buffered saline (PBS) pH7.4 at 1200 RPM, 4°C for 10 minutes. The resulting pellet was resuspended in cold PBS and washed twice at 800 RPM, 4°C for 10 minutes. PBMC at 1×10^6 /ml were allowed to adhere to plastic for 90 minutes at 37°C. The nonadherent cells were removed by 3 gentle washes with 5 ml warm PBS. This population will be referred to as 90 minute nonadherent cells in the text. Complete medium was added to the same dishes now containing adherent cells, and incubated for a further 16 hours at 37°C. Cells which detached during this period were collected by 3 consecutive washes with 5 ml warm PBS. In the text, the population collected after 16 hours is referred to as transiently adherent.

Purified T cells were obtained as follows: To deplete B cells, the 90 minutes nonadherent cells were applied to a nylon wool column and incubated for 30 minutes. The nonadherent cells were eluted by washing with 5x volumes warm PBS and subjected to a further 30 minute plastic adherence. The T cells obtained in this manner were 98% CD3⁺ positive as indicated by FACS analysis.

B cells were obtained by the following procedure: 90 minute nonadherent cells were incubated with Leu 4 (anti-CD3 mAb) and then treated with goat anti-mouse coupled magnetic beads (Dynal Inc. Great Neck, N.Y.) to remove T cells. The resulting population was subjected to plastic adherence for 30 minutes. The purity of the cell population was assessed by FACS analysis and found to be 98%⁺ for CD20.

Cell culture conditions: Transiently adherent cells were cultured in 0.2 ml volumes at 1×10^5 cells per well in flat bottom microtiter plates (NUNC). In some experiments, microtiter wells were coated with fibronectin, RGD peptide, collagen or HA as indicated above, prior to addition of cells. MAbs were added at the initiation of culture, at concentrations indicated in the text. PMA (10 μ M) was added at the initiation of culture. The cultures were incubated at 37°C and were analyzed daily for dendritic morphology using inverted phase microscopy.

Three-color Immunofluorescence staining procedure: Analysis of surface marker expression on the dendritic cell precursor population was performed by three-color immunofluorescence. Briefly, the cells were stained with the unconjugated antibody for 45 min., 4°C, and washed three times in PBS containing 1% FCS and 0.002% Na-azide. Biotin-conjugated goat anti-mouse antibody (Jackson, Westgrave PA) was added at 0.1 mg/ml for 30 min. at room temperature. Cells were washed twice and nonspecific Ig

binding sites blocked by adding mouse serum Ig (1mg/ml) for 10 minutes at room temperature. Cells were then labelled by adding Streptavidine-Duochrome (Becton Dickinson, San Jose, CA) together with FITC-and PE-conjugated antibodies, incubated for 20 min., 4°C, washed three times and fixed in 1% formalin.

Analysis of integrin expression was performed by two-color immunofluorescence. Transiently adherent cells were stained with unconjugated antibodies for 45 min., 4°C and washed three times in PBS containing 1% FCS and 0.002% azide. FITC-conjugated goat anti-mouse mAb was then added at 0.1 mg/ml for 30 min., 4°C, followed by three washes in PBS. Cells were then labelled with PE-conjugated antibodies for 30 min., 4°C, and washed three times in PBS. All experiments included staining with isotype matched control mAbs. Ten thousand cells were analyzed from each sample.

Analysis of three-color immunofluorescence data: Samples were analyzed on a FACSCAN (Becton Dickinson) using a single laser to excite FITC (green), PE (orange) and DUOCHROME (red). Red blood cells and dead cells were excluded by electronic gating on forward angle light scatter (FALS) and side scatter (SSC). All experiments included staining with isotype-matched control antibodies. Twenty thousand cells were analyzed from each sample. To evaluate the phenotype of CD9 positive cells within the population, electronic gates were set on basis of CD9 positivity, as well as on forward angle light scatter and side scatter (CD9 positive cells had different properties than the T cell subsets), and then analyzed for the two other colors in a given sample. To evaluate the phenotype of the CD9+ subset, files were gated either on basis of CD9 or CD19 positivity, since no CD19+ cells were CD9 negative, or gated on the population with high FALS and SSC properties. To analyze the two T cell subsets, files were gated on the basis of positivity for the relevant T cell marker (CD4 or CD8), or on low FALS and SSC properties. The CD4 and CD8 positive subsets had slightly different SSC properties; the CD8+ having a higher SSC than CD4+ cells, but still distinctively lower than CD9+ cells.

Cell Sorting: Transiently adherent mononuclear cells were stained with B4-FITC (CD19) and B1-PE (CD20), resuspended into PBS, and sorted into three populations: 1) CD19⁺CD20^{hi}, CD19⁺CD20^{low/-}, and CD19⁻CD20⁻ on a ELITE cell sorter (Coulter, Hialeah, Florida). The three sorted populations were collected into individual tubes containing FCS. All populations were centrifuged 10 minutes at 1500 rpm, resuspended in culture medium and plated in microtiter wells (NUNC), with or without anti-CD9 mAb.

Mixed lymphocyte cultures: Purified T cells in RPMI 1640 supplemented with 10% FCS, glutamine and streptomycin (hereafter referred to as complete medium), were cultured at 1.0×10^5 cells/ml in round-bottom multi-well plates (costar 3799 cambridge, MA). Mitomycin-treated transiently adherent cells, at concentrations ranging from 1×10^2 - 1×10^4 cells/ml, were added to the T cells at the start of culture.

Dendritic cells induced with anti-CD9 mAb were obtained at day 4 of culture and removed from tissue culture wells by treatment with 100ul lidocaine solution (0.7% lidocaine in HBSS), for 15 minutes at 37°C. The nonadherent cells were harvested and washed 2x in cold PBS to remove lidocaine, and resuspended in complete medium. Cells removed by the lidocaine solution were >95% viable as determined by trypan blue exclusion. These dendritic cells, at concentrations ranging from 1×10^2 - 1×10^4 , were added to purified T cells at the initiation of culture.

Cells were pulsed with 1 uCi of ³[H]-Thymidine (specific activity of 5 Ci/mmol) after 5 days of culture and harvested onto filter paper 6 hours later using a Titertek cell harvester. Radioactivity was counted in a Beckman LS7500 liquid scintillation counter.

C. RESULTS

1. Phenotypic characterization of the dendritic cell precursor population:

The transiently adherent cell population contained three major subsets of cells: 60-75% were CD9⁺ cells with a combined myeloid and activated B cell phenotype, 10-25% were CD4⁺ T cells while 15-30% were CD8⁺ T cells. Monocytes comprised less than 3%, and mature, resting B cells less than 1% of the total transiently adherent population. Forward angle and side scatter analysis indicated that cells in the CD9⁺ subset were larger in cell size and more granular, as compared to the two T cell subsets (Fig. 1).

This feature was used in gating for each of the subsets in the phenotypic analysis. The CD9⁺ subset, coexpressing the two B cell markers CD19 and CD20, included less than 5% positive CD4⁺ or CD8⁺ cells and did not express monocyte markers CD14 and CD15, nor the plasma cell marker PCA-1. The CD9⁺ subset strongly expressed HLA-Dr, CD44, CD45 and had heterogeneous expression of CD11b which included CD11b^{hi}, CD11b^{lo}, and CD11b⁻. CD9⁻ T cells and monocytes lacked HLA-Dr and the B cell markers CD19 and CD20 (Table 1 and Fig.2).

2. Anti-CD9 mabs induce development of dendritic cells from transiently adherent mononuclear cells:

Transiently adherent cells and 90 minute nonadherent cells, as well as purified T cells (98% CD3⁺) and purified B cells (98% CD20⁺), were cultured in flat bottom microtiter wells in the presence or absence of mAbs and morphological properties were monitored daily. By 48 hours of culture with anti-CD9 mAb 50H.19 IgG, 50H.19 F(ab')₂, or ALB6, the transiently adherent cells began to exhibit short cytoplasmic extensions. By 72 hours, cells with long cytoplasmic extensions (dendritic morphology) became evident among the transiently adherent cells (Fig.3a), but not among purified T or B cells or 90 minute nonadherent cells (<1%). The development of dendritic cells in culture was specific for

anti-CD9 mAbs, as the addition of mAbs directed against HLA-Dr, CD44 or CD11b to cultures of transiently adherent cells, failed to induce development of dendritic cells (Fig.3b-d) even though these surface markers were abundantly expressed as indicated by FACS analysis. Addition of 10 μ M PMA at the initiation of culture promoted less than 10% of transiently adherent cells to develop dendritic morphology (Fig.3e), indicating that the activation of PKC is insufficient to induce DC differentiation. Direct cell counting of cultures containing anti-CD9 mAbs indicates that 30-60% of the cells within the transiently adherent population developed dendritic cell morphology. However, this preparation of dendritic cells represents no greater than 1% of the total isolated PBMC's.

Cytochalasin B, which prevents actin polymerization, and hydroxyurea which inhibits DNA synthesis were added at the initiation of culture in order to determine whether cytoskeleton assembly, or cellular proliferation were required for dendritic cell development. Transiently adherent cells cultured with cytochalasin B did not develop dendritic cells, even if cultured for extended periods of time. However, cells cultured with hydroxyurea did develop dendritic cells suggesting that DNA synthesis is not required for differentiation.

3. CD19⁺CD20^{lo/-} cells give rise to dendritic cells:

Transiently adherent cells were sorted into CD9⁺CD19⁺CD20^{hi}, CD9⁺CD19⁺CD20^{lo/-} and CD9⁻CD19⁻CD20⁻ populations (Fig.4a) CD9⁺CD19⁺CD20^{hi} and CD9⁺CD19⁺CD20^{lo/-} were analyzed to determine whether either of these populations contained DC precursors. Side scatter profiles of the sorted populations show that the CD20^{lo/-} population were larger and more granular cells as compared to the CD20^{hi} population (Fig. 4b).

The CD9⁺CD19⁺CD20^{hi} and CD9⁺CD19⁺CD20^{lo/-} sorted cells were incubated with 50H.19 and monitored daily for dendritic cell morphology. By 72 hours, dendritic cells were seen to arise within the CD9⁺CD19⁺CD20^{lo/-} population, but not from the CD9⁺CD19⁺CD20^{hi} population (Fig.5).

4. Adhesion receptors expressed by transiently adherent cells:

Integrin expression on the CD9⁺ sorted populations (CD9⁺CD19⁺CD20^{lo/-} vs. CD9⁺CD19⁺CD20^{hi}) was analyzed. As shown in Table II, the CD9⁺CD20^{lo/-} population strongly expressed $\alpha 1\beta 1$, $\alpha 2\beta 1$, $\alpha 3\beta 1$ and $\alpha 5\beta 1$, moderately expressed $\alpha 4\beta 1$ and dimly expressed $\alpha 6\beta 1$. The CD9⁺CD19⁺CD20^{hi} population strongly expressed $\alpha 3\beta 1$ and $\alpha 5\beta 1$, moderately expressed $\alpha 1\beta 1$, $\alpha 2\beta 1$, $\alpha 4\beta 1$ and had low expression of $\alpha 6\beta 1$. Both populations were greater than 90% positive for $\beta 1$, LFA-3, CD11a, CD11b and CD44, but did not express CD11c or $\beta 3$ integrins. Expression of $\beta 7$ included 80% positive CD9⁺CD19⁺CD20^{lo/-} and 26% positive CD9⁺CD19⁺CD20^{hi} (Table II). CD1, a surface receptor present on langerhans cells, was found on CD9⁺CD19⁺CD20^{hi} cells, but was expressed on a small subpopulation of CD9⁺CD19⁺CD20^{lo/-} cells.

5. Function of adhesion receptors on the adherent dendritic cell:

It was observed that the latter stages of dendritic cell development was characterized by cells exhibiting long cytoplasmic processes which were strongly adherent. To determine whether specific cell:matrix interactions were a requirement, the transiently adherent cells were cultured on various ECM components with or without anti-CD9 mAbs. Dendritic cells developed efficiently when transiently adherent cells were cultured on fibronectin, but not when they were cultured on a matrix consisting of collagen, laminin, collagen/laminin together, or hyaluronan. The development of dendritic cells on fibronectin could be

blocked by synthetic peptides containing the RGD sequence, but not by a synthetic peptide homologous to the CS-1 fibronectin domain (Table III). These results suggest the involvement of fibronectin, and RGD dependent receptors such as $\alpha 5\beta 1$, in dendritic cell development.

The $\beta 1$ integrins $\alpha 4\beta 1$ and $\alpha 5\beta 1$ bind to fibronectin via specific sequences (22), and we wished to determine whether these recognition sequences were involved in promoting the adhesive characteristic of the anti-CD9 mAb induced dendritic cells. Transiently adherent populations were plated in microtiter wells coated with peptide containing the $\alpha 5\beta 1$ recognition sequence RGD. Addition of mAb 50H.19 or ALB6 to transiently adherent cells cultured on RGD peptide promoted development of the dendritic cells as early as 24 hours after addition of mAb (Fig. 6a). However, dendritic cells were not seen in the absence of anti-CD9 mAbs. The development of the dendritic cells could be blocked by the addition of 0.25-1.0 mM soluble RGD peptide, added at the initiation of culture (Fig. 6b). A functional role for $\alpha 5\beta 1$ could be demonstrated by the use of blocking mAbs directed against either the $\alpha 5$ or $\beta 1$. Addition of less than 2.5 $\mu\text{g/ml}$ mAbs P1D6 or 4B4, (against $\alpha 5$ and $\beta 1$ chains respectively), at the initiation of culture, reduced the anti-CD9 mAb induced development of dendritic cells to less than 10% dendritic cells (Fig. 6c). However, mAbs 44H6, P1E6 or HP2/1 directed against $\alpha 4$ were unable to reduce dendritic cell numbers. The $\alpha 4$ subunit can form a heterodimer with the $\beta 7$ integrin subunit (21). $\beta 7$ is expressed on the transiently adherent cells and we wished to determine whether the $\alpha 4\beta 7$ heterodimer was involved in dendritic cell development. The anti- $\beta 7$ mAb Act-1 was unable to block anti-CD9 mAb induced dendritic cell development. Mab 50B4, directed against CD44, completely abrogated development of dendritic cells. Addition of 2 $\mu\text{g/ml}$ 50B4 was sufficient to reduce anti-CD9 mAb induced dendritic cell numbers to less than 1%. These results suggest that $\alpha 5$ recognition of fibronectin at the RGD domain, and

CD44 recognition of a ligand other than HA, is involved in anti-CD9 induced dendritic cell development.

6. *Anti-CD9 induced dendritic cells stimulate MLR:*

Dendritic cells are potent stimulators of T cells (22). We compared both the transiently adherent cells and anti-CD9 mAb induced dendritic cells, for their ability to stimulate T cells in an MLC. The transiently adherent cells were able to stimulate an MLR with as few as 1×10^2 cells/well (Fig. 7).

D. DISCUSSION

In this chapter, we present evidence that antibody modulation of CD9 can induce development of cells with dendritic morphology. Our studies focused on the functional and phenotypic characterization of a CD9⁺ population within peripheral blood in order to identify a dendritic cell precursor. The CD9⁺ precursor cells express the pan B cell marker CD19, exhibit low expression of CD20, and do not express the plasma cell marker PCA-1. The CD9⁺ cells are positive for CD11b, a marker present on cells of myeloid origin which has also been observed on resting and activated B cells in both mice and human (23, 24). The CD9⁺ cells also express low amounts of CD5, and strongly express CD44, both which are found on B cells at various stages of differentiation and activation (23, 25-27). The expression of CD19, together with CD5, CD44 and CD11b, may indicate an activated B cell subset. The success in obtaining dendritic cells from a sorted population co-expressing CD9 and CD19 further supports the precursor being of B cell origin. The CD9⁺CD19⁺ dendritic cell precursor only weakly express CD20. CD19 is a panB cell marker, and is expressed at the earliest stages of B cell differentiation (28). Expression of CD20 denotes a more mature stage of B cell differentiation, and its expression is inversely

related to the expression of early B cell differentiation antigens such as CD9, CD10 and TdT (29). Activation of B cells does not appear to change the expression of CD19, but does appear to downregulate the expression of CD20 (30). CD9 is upregulated upon B cell activation (31). The expression of CD9 and CD19, and the lack of CD20, suggests that the peripheral blood cell described herein may represent a B lineage cell which deviated from the classical pathway of B cell differentiation at an early stage, prior to the expression of CD20, or may represent a late B cell which has lost CD20 expression.

Since the appearance of cells with the dendritic morphology is increased by culturing with anti-CD9 antibody, we believe that the dendritic precursor cells are CD9 positive. However, as the transiently adherent population contains more than one cell type, the possibility of an indirect mechanism acting upon a CD9 negative population cannot be excluded. It has been reported that a proportion of CD8⁺ and CD4⁺ activated T cells express CD9 (32), and this activation may have occurred *in vivo* in a small fraction of peripheral blood T cells. That the dendritic cells were found in cultures in the presence of hydroxyurea suggests that these cells did not arise from an outgrowth of a small population of cells. As the CD9⁺CD4⁺ and CD9⁺CD8⁺ cells represent less than 5% of the starting population (Table I), it is unlikely that these cells could give rise to the 60% dendritic cells observed in some cultures, in the absence of cellular proliferation. Furthermore, purified populations of CD3⁺ T cells failed to give rise to dendritic cells when cultured with anti-CD9 mAbs. Given the above findings, it is unlikely that the small proportion of T cells present within the transiently adherent cells, or any T cell contaminants within the sorted CD9⁺CD19⁺CD20^{lo/-} population, could give rise to dendritic cells.

Although the dendritic cell precursors express multiple adhesion receptors, our results suggest that recognition of the fibronectin central cell binding domain (CCBD) by $\alpha 5\beta 1$, as well as interactions mediated by CD44, is critical in the differentiation process towards an

adherent morphology. Using a panel of mAbs, we have shown that only mAbs directed against $\alpha 5$ or $\beta 1$, and a mAb against CD44, were able to block development of DC. Furthermore, of the various purified ECM components used in this study, only fibronectin and a synthetic peptide containing the RGD sequence, supported dendritic cell differentiation. Finally, a soluble RGD containing peptide, but not a peptide analogous to the CS-1 domain of fibronectin, blocked dendritic cell differentiation. CD44, a receptor for both ECM components and cell surface receptors, has been implicated in lymphocyte homing and interactions crucial to lymphocyte precursor differentiation (33). That the anti-CD44 mAb 50B4 was a potent inhibitor in this study suggests that CD44:ligand interactions are equally important to dendritic cell development. CD44 has been shown to bind to hyaluronan (34) and collagen (35) and to a ligand on high endothelial venules (HEV) (36). Both collagen and HA were not able to support anti-CD9 induced dendritic cell, suggesting that CD44 may participate in dendritic cell development by recognizing a ligand on HEV, or an as yet unidentified ECM component.

We have reported the phenotype of a dendritic cell precursor present in peripheral blood, and its differentiation towards an adherent cell with dendritic morphology. That the dendritic cell precursors initially developed from an adherent population that became nonadherent (transiently adherent) suggests a differentiation process which is marked by altered adhesive states and possibly involves multiple adhesion receptors. The greatly increased development of adherent cells in the presence of CD9 suggests that dendritic cell adherence proceeds through a mechanism involving CD9 regulation of $\alpha 5\beta 1$, and possibly regulation of CD44, ligand recognition.

Number of Cells

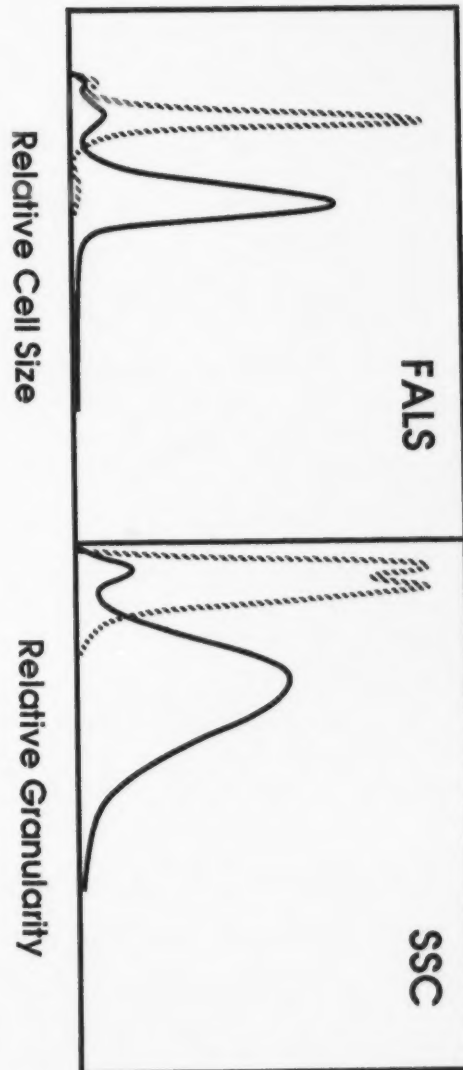


Figure 1. *Transiently adherent cell population has increased cell size and intracellular granularity compared to lymphocytes.* Forward angle scatter (FALS) and side scatter (SSC) was analyzed on a fluorescence activated cell sorter. The cells were gated on basis of fluorescence for either CD4/CD8 (dotted line) or for CD9 (full line). FALS and SSC are given on a linear scale.

Figure 1

Figure 2

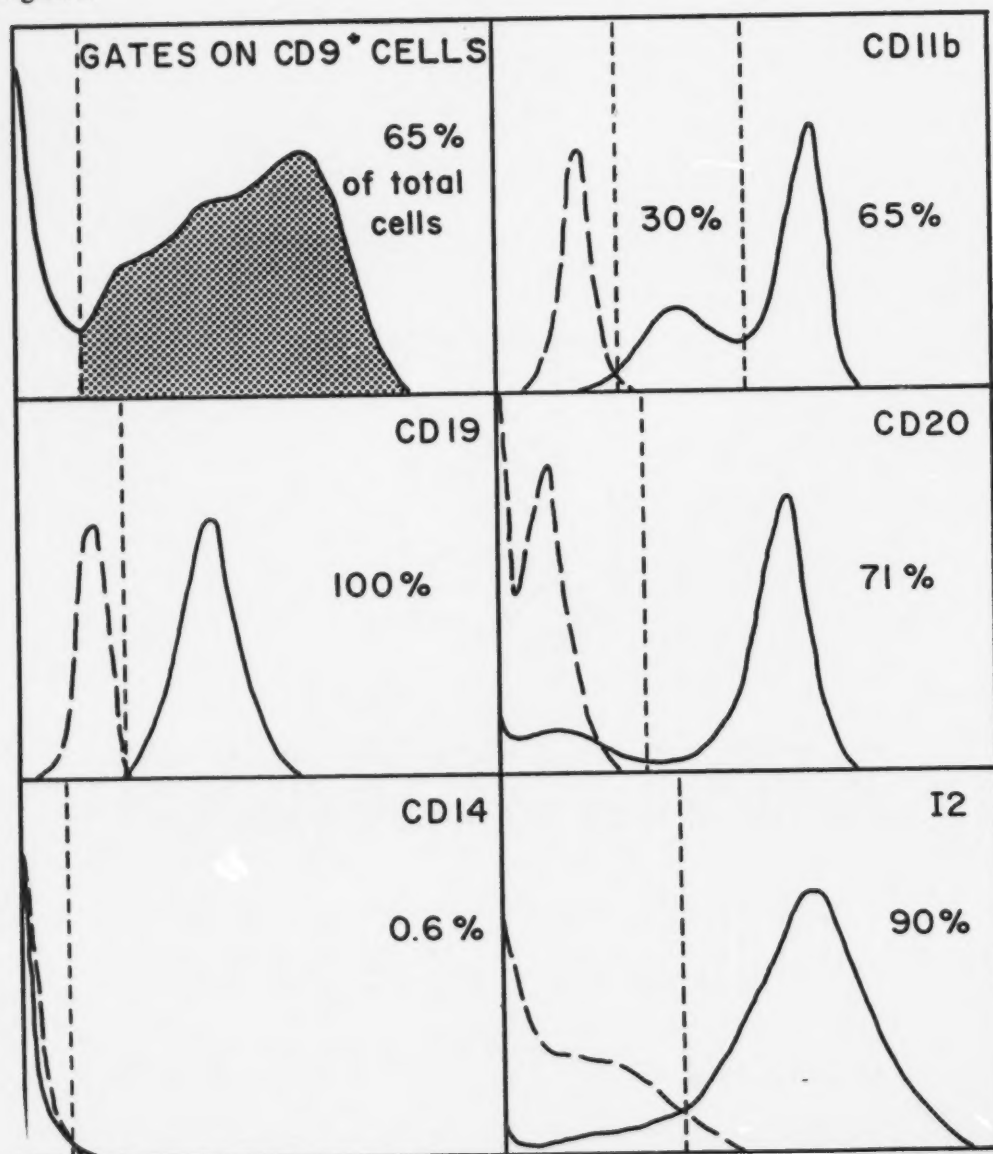


Figure 2. Phenotype of CD9⁺ transiently adherent cells. Transiently adherent cells were isolated as indicated in materials and methods and were stained for the following markers using directly PE-coupled mAbs as indicated in materials and methods: CD20, CD19, HLA-DR, CD11b, CD4, and CD8. The transiently adherent cells were stained for the above markers and analyzed for CD9 using 50H.19 followed by goat anti-mouse FITC coupled IgG, as indicated in materials and methods. The transiently adherent cells were gated on the CD9 positive cells and the CD9 positive population was analyzed for the above markers.

Figure 3

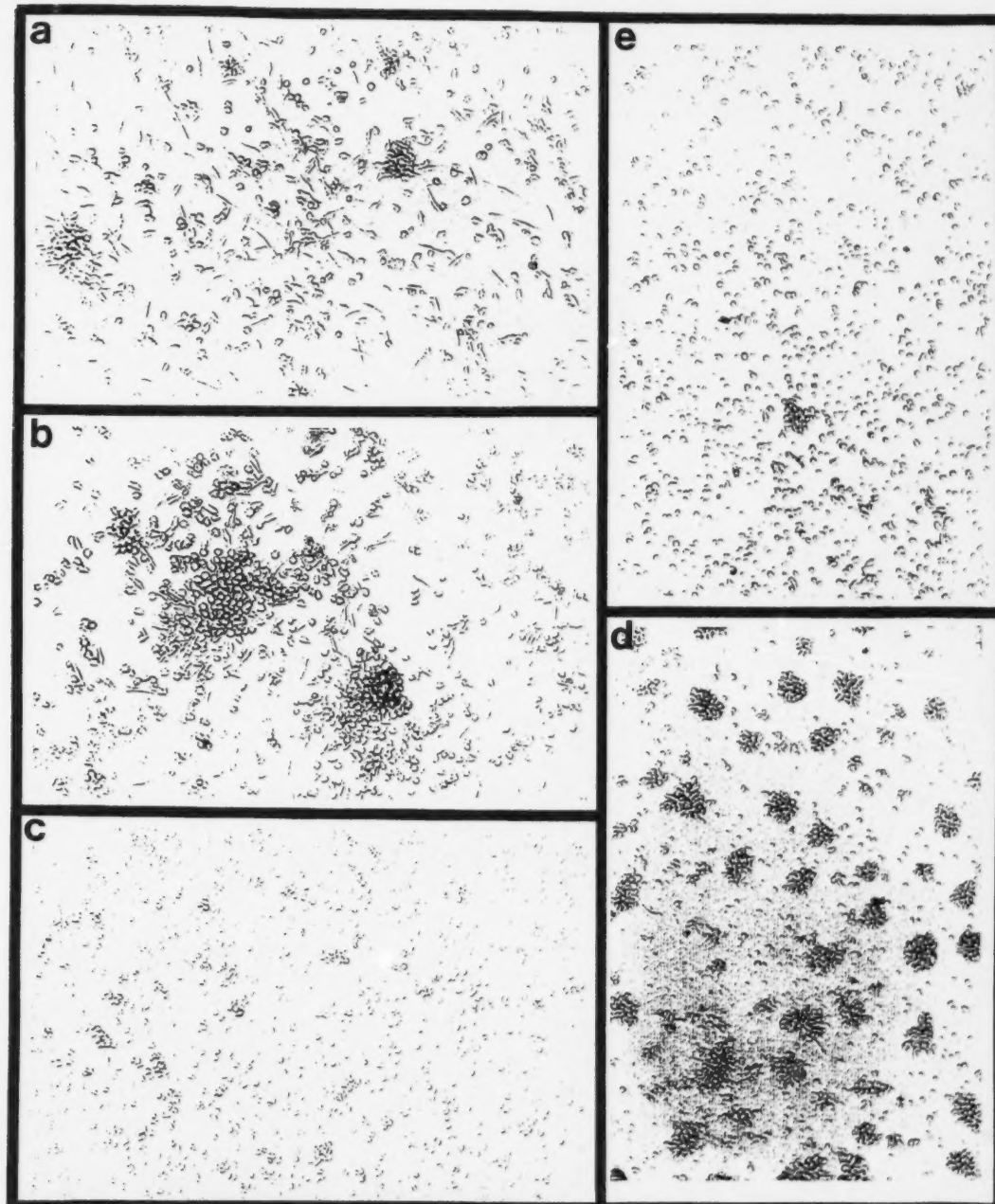


Figure 3. *Development of dendritic cells by anti-CD9 mAb.* Transiently adherent cells were cultured with (a) 1 ug/ml 50H.19, (b) 1 ug/ml 7H.3, (c) 0.5 ug/ml 50B4, (d) 1 ug/ml Mo-1 or (e) 10 uM PMA as indicated in materials and methods and photographs taken at 96 hours of culture.

Figure 4

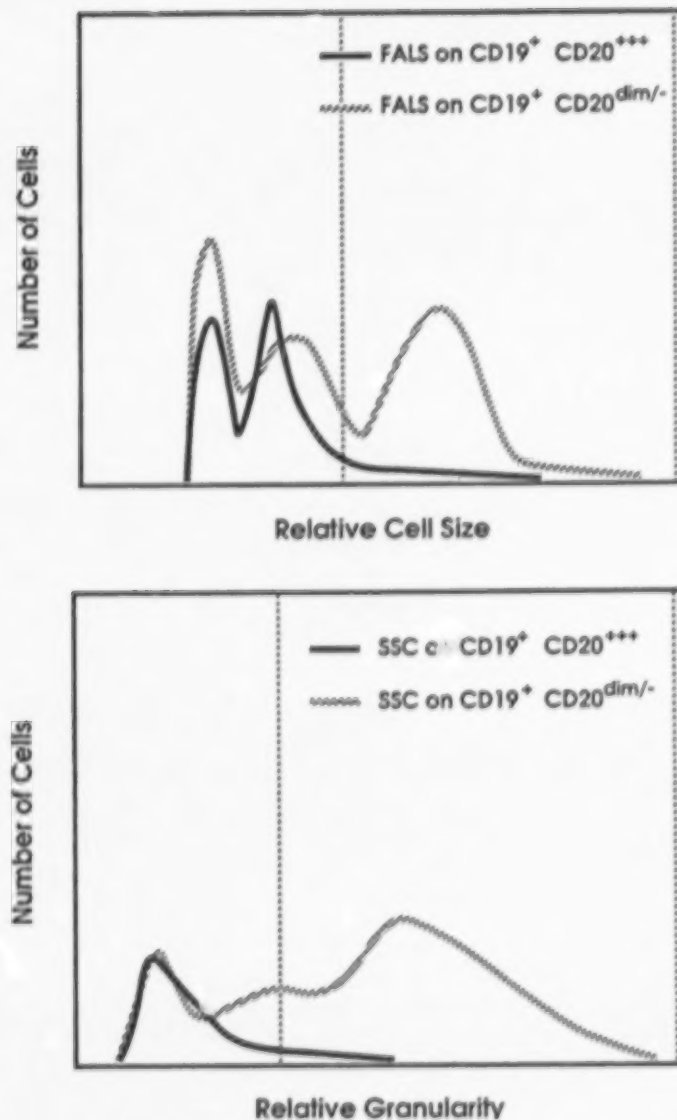


Figure 4. *Forward and side scatter analysis of sorted CD9⁺ precursor cells.* Transiently adherent cells were sorted into CD19⁻CD20⁻, CD19⁺CD20^{lo/-} and CD19⁺CD20^{hi} cells. The sorted populations were cultured with anti-CD9 mAbs (1 μ g/ml 50H.19) and the development of dendritic cells observed. (b) The CD19⁺CD20^{lo/-} and CD19⁺CD20^{hi} sorted populations were analyzed for forward and angle light scatter and side scatter.

Figure 5

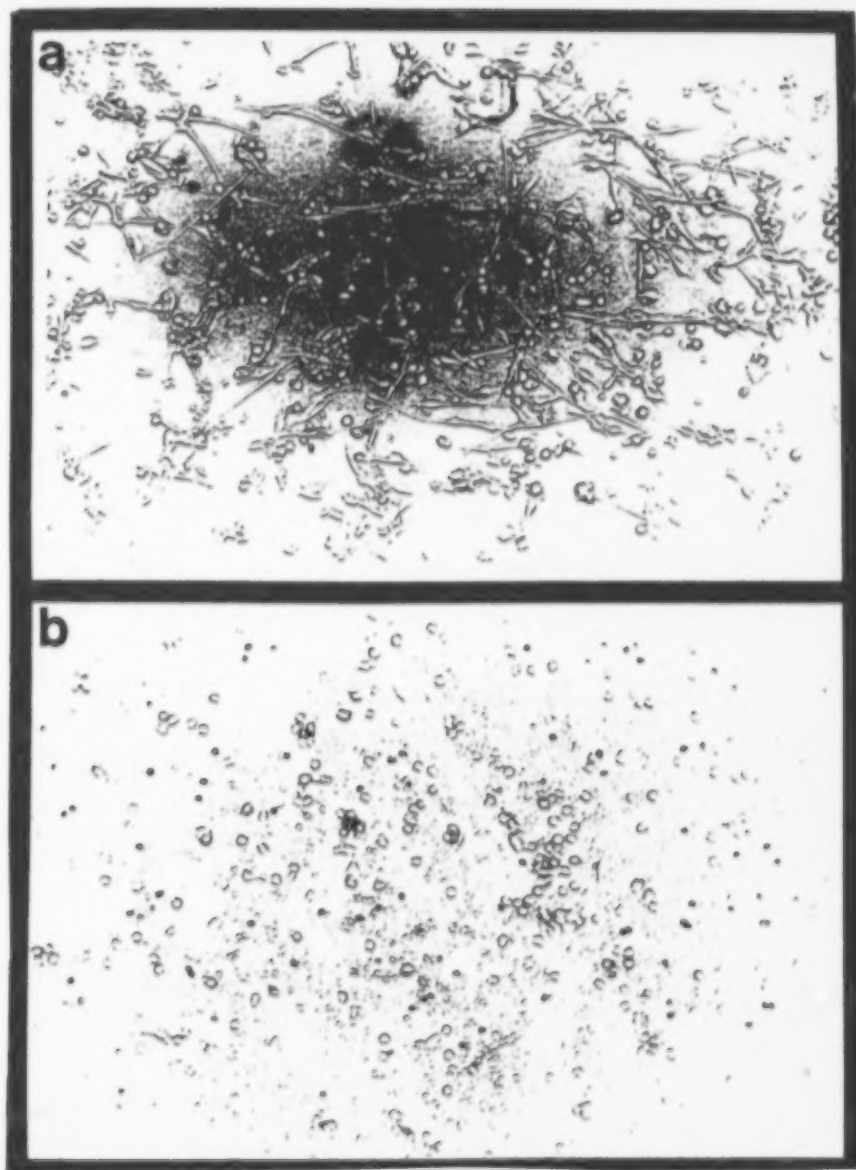


Figure 5. *CD19+CD20^{lo/-} but not CD19+CD20^{hi} give rise to dendritic cells.* CD19+CD20^{lo/-} cells (A) and CD19+CD20^{hi} (B) cells were isolated and incubated with 1 μ g/ml 50H.19. Cultures were analyzed daily for appearance of dendritic cells. The addition of 1 μ g/ml irrelevant IgG α control mAb did not promote dendritic cell development.

Figure 6

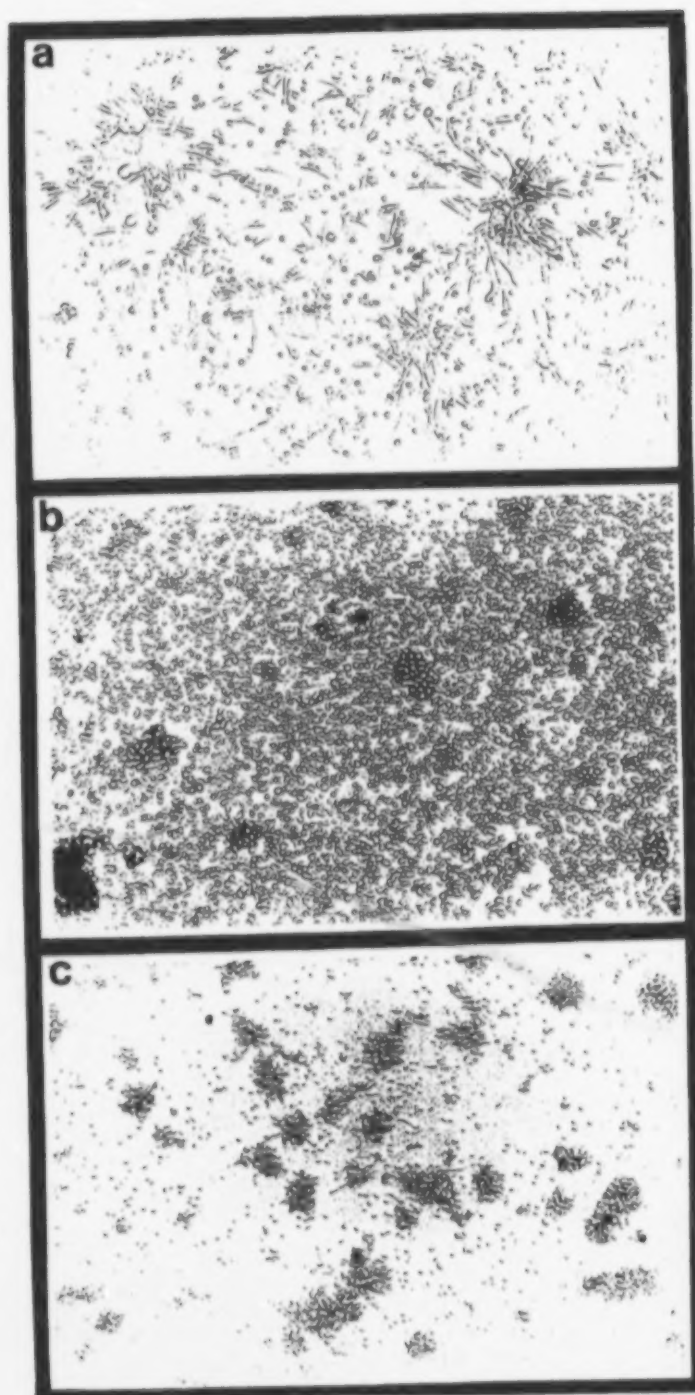


Figure 6. Role of extracellular matrix and extracellular matrix receptors in dendritic cell development. Transiently adherent cells were isolated and incubated on matrices composed of 25 ug/ml RGD peptide (a), and cocultured with 1mM soluble RGD peptide (b), or 2.5 ug/ml anti- $\alpha 5 \beta 1$ mAb PID6 (c). Anti-CD9 mAb 50H.19 (1ug/ml), or irrelevant IgG2a mAb was added at the initiation of culture.

Figure 7

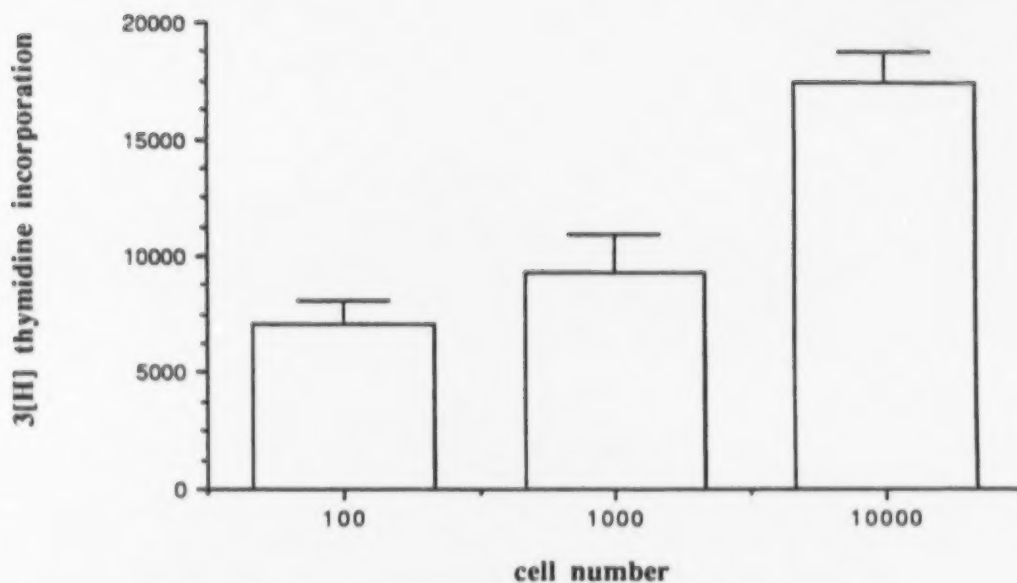


Figure 7. *Dendritic cell precursors are potent stimulators of mixed lymphocyte reactions.* Transiently adherent cells were isolated as indicated in materials and methods and treated with mitomycin C to inhibit their proliferation. Mitomycin C treated cells were added to autologous purified T lymphocytes and T cell proliferation was measured at day 5 by incorporation of ^3H thymidine. Figure is representative of 4 experiments. Background CPM did not exceed 300 and has been subtracted.

TABLE I: PHENOTYPE OF TRANSIENTLY ADHERENT POPULATION^{a)}

	CD9	CD19	CD20	CD21	PCA-1	CD44	CD45	I2	CD11b	CD14	CD15	CD5	CD4	CD8
transiently adherent	++b)	++	++	-	-	+++	++	++	-/++/+++	-	-	+	-	-
CD4 ⁺ subset	-	-	-	-	-	-	+	-	-	-	-	+	+++	-
CD8 ⁺ subset	-	-	-	-	-	-	+	-	+	-	-	++	-	+++
monocytes	+++	-	-	-	-	nt	nt	+	+++	+++	++	-	-	-

a) as determined over 4 experiments

b) +++ brightly positive
 ++ moderately positive
 + dim, but positive
 - less than 5% positive

TABLE II: Adhesion receptors expressed by CD9⁺CD19⁺CD20⁺ vs CD9⁺CD19⁺CD20⁺ cells

Adhesion receptors	Percent cells positive	
	CD9 ⁺ CD19 ⁺ CD20 ^{lo/-} (high SSC/FALS)	CD9 ⁺ CD19 ⁺ CD20 ^{hi} (low SSC/FALS)
$\alpha 1$	87% hi	30%
$\alpha 2$	82% hi	35%
$\alpha 3$	80%	20%
$\alpha 4$	60%	30%
$\alpha 5$	>90% hi	25% lo
$\alpha 6$	30% dim	20% lo
$\beta 1$	98%	30%
$\beta 2$	98% hi	90% hi
$\beta 3$	3%	5%
$\beta 7$	80%	35% lo
LFA-3	98 %hi	nt
CD11c	5%	nt
CD1	98%	34%

Transiently adherent cells were incubated with mAb against the adhesion receptors indicated above, or with irrelevant Ig isotype control mAbs. Positive reactivity was determined by FACS. The results are representative of 3 experiments.

TABLE III: Dendritic cell development on fibronectin matrices can be blocked by RGD, but not CS-1 peptides.

<u>ECM protein</u>	<u>capacity to support DC development</u>
collagen	no
laminin	no
collagen/laminin	no
hyaluronin	no
fibronectin	yes
fibronectin/RGD	no
fibronectin/RGE	yes
fibronectin/CS-1	yes
fibronectin/CS-1s	yes

Dendritic cell precursors, obtained as indicated in materials and methods, were plated in wells coated with collagen (50ug/ml), laminin (5ug/ml), collagen and laminin, hyaluronin (0.1-5ug/ml) and fibronectin (25ug/ml). The anti-CD9 mAbs 50H.19 and ALB₆ were added at the initiation of culture and the development of dendritic cells was observed over 4 days. The development of dendritic cells on the above matrices was compared with or without anti-CD9 mAbs, and compared to anti-CD9 mAb induced development of dendritic cells in the absence of matrices. In some experiments cells plated on fibronectin matrices were pre-treated with 500ug/ml CS-1 and CS-1s control peptide, 30ug/ml RGD (Gly-Arg-Gly-Asp-Thr-Pro) or RGE control peptide. Results were obtained from 4 experiments.

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GENERAL DISCUSSION AND SUMMARY

The objective of this research project was to determine whether modulation of CD9 by mAbs resulted in adhesive events in cells other than platelets. This work is based on the premise that CD9 regulates adhesive interactions, possibly mediated by members of the integrin family of adhesion receptors, which are important to cell differentiation.

Evidence demonstrating an adhesive function for CD9 stems from studies utilizing a platelet system, and as discussed in the introduction, anti-CD9 mAbs initiate platelet activation and aggregation. My primary focus was to develop an adhesion assays to observe aggregation of nucleated cells. Different cell CD9⁺ hematopoietic cells, such as monocytes, activated T cells and activated B cells were considered in an attempt to obtain the most useful system in which to observe the adhesion promoting capacity of anti-CD9 mAbs. Monocytes were excluded as a relevant cell type for a variety of reasons which include the following: 1) it is unclear whether CD9 expressed on monocytes is a result of acquisition from cell surface platelets, or whether monocytes synthesize the antigen, 2) the purification of monocytes includes adhesion to plastic or glass, which may affect interpretation of final results, 3) the use of EDTA to reduce platelet adhesion to monocytes also disrupts integrin function. The use of T cells or B cells also had inherent problems as only subpopulations of peripheral blood T and B cells weakly express the antigen. However, CD9 expression is increased upon activation on a subpopulation of both T cells and B cells. While efforts were made to observe CD9 function with primary activated T or B cell lines, observations were inconsistent as the longevity of the lines varied. The lack of relevant CD9 positive T or B cell lines, and our inability to induce CD9 expression on these negative lines, was yet another reason to look further for a relevant cell assay system.

As indicated in chapter I, pre-B cell (CD19⁺CD10⁺TdT⁺) within bone marrow are CD9 positive, as are cells from non-T, non-B acute lymphoblastic leukemia (ALL) patients. Pre-B ALL cell lines are readily available and express CD9 at high concentrations suggesting that CD9 may play a functional role in these cells. Thus pre-B cells were chosen as a model cell type with which to observe CD9 function. Pre-B ALL cell lines were used throughout this study rather than normal pre-B cells obtained from marrow, or fresh pre-B ALL cells due to the difficulty in obtaining such materials. However, that normal pre-B cells, pre-B ALL cell and pre-B cell lines exhibit similar adhesive qualities² allows the use of pre-B cell lines as a model system in which to examine pre-B cell adhesion. In chapter II, evidence is presented that anti-CD9 mAbs do induce aggregation of pre-B cells, and that CD9 induced adhesion was independent of β_2 integrins. This observation indicated that a molecule other than LFA-1, a β_2 integrin proposed to mediate many lymphocytic adhesion events, was involved in lymphocyte adhesion. While LFA-1 was shown not to be involved in homotypic adhesion induced by anti-CD9 mAbs, the molecules which mediate this phenomena are not recognized. I was unable to inhibit adhesion with mAbs directed against α_4 , α_5 , or β_1 suggesting that these integrins do not mediate CD9 induced homotypic adhesion. Indeed, as indicated in chapter III, addition of mAbs against α_4 , α_5 , or β_1 induced homotypic aggregation of five pre-B cell lines. The capacity of anti-integrin mAbs to induce aggregation correlated with the expression of CD9, suggesting that CD9 may function downstream of the integrin:ligand interaction. Although the molecules which mediate homotypic cell adhesion induced by either anti-CD9 mAbs, or anti-integrin mAbs, were not defined they may represent adhesion receptors, or other cell surface components, not tested for in this study. In chapter IV anti-CD9 mAbs enhance heterotypic adhesion between pre-B cells and cell surface bound fibronectin through the upregulation of integrins. The data

² Ryan, D. H., et al. 1992. Blood suppl. 80. 1:36A

presented in chapter III and IV may suggest that heterotypic and homotypic interactions are unique phenomena mediated through different types of adhesion molecules (integrins vs. unknown). Thus, CD9 may represent a family of molecules with the capacity to upregulate a wide variety of adhesion receptors. The mechanism by which CD9 may regulate adhesion proteins is unclear however, the strict requirement for CD9 in anti-integrin mAb induced aggregation suggests an importance for CD9 induced signal transduction (inside-out pathway). The upregulation of integrin avidity by CD9 (chapter IV) may occur through an inside-out signaling mechanism, or may also occur through a direct interaction of CD9 with β_1 integrins to alter integrin conformation (out-side in pathway) and upregulate ligand interactions.

In chapter IV, I have shown that anti-CD9 mAbs enhance pre-B cell binding to bone marrow fibroblasts through the upregulation of $\alpha_4\beta_1$ and $\alpha_5\beta_1$ binding to cell surface bound fibronectin, implicating CD9 as a regulator of cell adhesion mediated by integrins. That CD9 specifically upregulates $\alpha_4\beta_1$ and $\alpha_5\beta_1$ adhesion to an extracellular matrix protein (fibronectin), and does not alter $\alpha_4\beta_1$ avidity for VCAM-1, it is intriguing to suggest that CD9 may upregulate the avidity of a subset of $\alpha_4\beta_1$ receptors. While integrins can recognize multiple ligands, their interactions can be dictated by the α - β heterodimer pairing (eg. $\alpha_v\beta_3$ vs. $\alpha_v\beta_5$), state of cell activation, divalent cations, and lipid factors. Integrin specificity may be further dictated by cell-specific expression, suggesting cell-specific regulatory mechanisms. The data presented in this study does suggest that pre-B cells regulate adhesive interactions through a CD9-dependent pathway, a mechanism which may not be present in circulating CD9^{-/-} T cells or B cells

CD9 is a member of a newly described four-pass family of type-three integral transmembrane proteins. It is suggested that members of this family play a role in cell proliferation and differentiation, however the mechanisms by which they function is

unclear. The analysis of CD9 function is limited by its stage and cell type specific expression. While other members of this family are more broadly expressed (see chapter I) the study and comparison of these proteins may provide information on their function. TAPA-1 (described in Chapter I) appears most similar to CD9 both biochemically and functionally. Anti-TAPA-1 mAbs induce aggregation of lymphoid cell lines in a manner similar to anti-CD9 mAb indicating that both these molecules are capable of signal transduction. In comparison to the CD9-GpIIb/IIIa association, TAPA-1 has been found to be associated with Leu-13 (chapter I), and more recently with the CD19/CD21 B lineage-specific signal transduction complex¹. The authors suggest that the CD19/CD21 complex may regulate the function of TAPA-1, and further suggest that other signaling complexes on different cell types may provide cell type-specific regulation of TAPA-1. If one supposes that members of the four-pass protein family play analogous roles, the data presented in this study suggests that members of the four pass family may be components of large signaling complexes (eg. TAPA-1/Leu-13 and CD19/CD21 or CD9 and $\alpha 4\beta 1/\alpha 5\beta 1$), to which they contribute unique signalling potentials, and serve to bring together proliferation and adhesion signals. Indeed, with respect to their structural similarities to ion channels, it is tempting to speculate that these proteins could function as receptor gated ion channels. The implications of such a notion would be relevant to many cellular activities, including cell activation, growth, and adhesion.

Integrins receptors may serve not only as receptors which link the cell cytoskeleton to the extracellular matrix, but may also act as signaling receptors and influence not only cell motility and migration, but also cell growth and differentiation. As indicated in this study, CD9 regulates adhesive events of pre-B cells, as well as dendritic cell precursors. That CD9 is capable of influencing cell adhesion indicates a commonality with the integrin signaling pathway. Although the mechanism by which CD9, and other members

¹ Bradbury, L. E., et al. 1992. *J. Immunol.* 149:2841.

of the four-pass transmembrane family influence cell adhesion and proliferation is largely unknown, evidence that CD9 associates with GpIIb/IIIa and that TAPA-1 associates with CD19/CD21 suggests that the four-pass proteins may be involved in the regulation of signals, initiated through other surface receptors such as CD19/CD21, or may initiate the transduction of intracellular signals which regulate other receptors, such as integrins. The data provided in this study suggests that CD9 may initiate transduction of intracellular signals which ultimately lead to adhesion mediated by integrin receptors.